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OF
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(THE PUBLICATION OF THE MEDICAL SCHOOL AND HOSPITAL)

(SUPPORTED BY THE DE LAMAR FUND OF THE JOHNS HOPKINS UNIVERSITY)

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January, 1943

Pyridoxine Deficiency in Swine, With Particular Reference to Anemia, Epileptiform Convulsions and Fatty Liver. Maxwell M. Wintrobe, Richard H. Follis, Jr., Mitchell H. Miller, Harold J. Stein, Raul Alcayaga, Stewart Humphreys, Adolph Suksta and George E. Cartwright 1

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PYRIDOXINE DEFICIENCY IN SWINE

With Particular Reference to Anemia, Epileptiform Convulsions and Fatty Liver

MAXWELL M. WINTROBE, RICHARD H. FOLLIS, JR., MITCHELL H. MILLER,[1] HAROLD J. STEIN,[2] RAUL ALCAYAGA,[3] STEWART HUMPHREYS, ADOLPH SUKSTA AND GEORGE E. CARTWRIGHT

From the Departments of Medicine and Pathology, Johns Hopkins University

Received for publication October 27, 1942

We have shown (1) that good growth and development take place in young pigs when they are fed a diet poor in the vitamin B complex if the diet is supplemented with crystalline thiamine, riboflavin, nicotinic acid, pyridoxine, choline, and calcium pantothenate. This has made it possible to observe the effects produced by the total or partial omission of each of these crystalline vitamins without the risk of the confusion which is inevitable when crude extracts and fractions, or autoclaved diets are used for the study of deficiencies of the vitamin B complex. We have described elsewhere the effects of thiamine deficiency in swine (2,3,4) and have also reported that the sensory neuron degeneration which develops in young pigs as the result of a deficiency in the vitamin B complex (5,6,7) occurs when there is a lack of pyridoxine and of calcium pantothenate and when either one of these vitamins is not furnished (1).

It is the purpose of this communication to describe the effects produced when pyridoxine is omitted from the mixture of crystalline vitamins and to discuss particularly the anemia, the hemosiderosis, the convulsions and the fatty liver which result.

REVIEW OF LITERATURE

Chick and her co-workers (8), in the only study in pigs which seems pertinent here, reported impaired growth when a diet consisting of puri-

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fied casein, purified starch, cottonseed oil, cod liver oil and salt mixture was supplemented only with crystalline thiamine, riboflavin and nicotinic acid. It was shown that the animals required substances present in eluate and filtrate fractions of liver as well as some factor present in yeast. When the basal diet was supplemented by eluate only, a flaccid paralysis of the hindquarters developed; when only the filtrate was given a microcytic anemia and "typical epileptic fits" were observed. Administration of the eluate, which was known to contain pyridoxine, relieved the anemia and caused the fits to cease. It was noted that the response of the blood was slow whereas growth and, presumably, increase in total blood volume, was rapid. In the pigs lacking the filtrate fraction normocytic anemia resembling that previously described by us (5,9) was observed. Chick and her co-workers concluded that "there is more than one substance in the vitamin B_2 complex, the lack of which embarrasses the blood-forming tissues."

In seven puppies Fouts et al. (10) reported hypochromic microcytic anemia, accompanied by convulsive fits, which was cured when an extract of rice polishings containing the "rat anti-dermatitis factor" was added to the diet. The basal diet included vitamins A and D, thiamine, riboflavin and a liver extract containing the chick anti-dermatitis factor and nicotinic acid. The response to treatment in two dogs was accompanied by a reticulocyte rise reaching a peak on the second and third days, respectively, after treatment was commenced, and tripling of the hemoglobin content of the blood in 4 to 12 days, respectively. It is of interest that convulsions increased in severity in one dog in spite of rapid blood regeneration in response to treatment, and led to the death of this animal. The bone marrow was found normal at autopsy. In another dog which was not treated the bone marrow was red and hyperplastic and consisted chiefly of normoblasts. The spleen was enlarged and quite soft. The liver was filled with more than the usual amounts of fat. In a later report (11) these authors noted in three adult dogs failure of the anemia to respond to treatment with iron and ammonium citrate together with copper sulfate, and prompt improvement following oral or intravenous administration of crystalline factor I (rat antidermatitis factor, vitamin B_6) in amounts of 60 μg. per kg. body weight. It was also noted that the serum-iron was abnormally high at the time of severe anemia.

Borson and Mettier (12) observed a prompt response of the microcytic

anemia when synthetic B_6 was given 4 adult dogs which had been fed a diet deficient in this vitamin but they found that blood regeneration ceased after a time and normocytic anemia developed. The latter could be relieved by giving a liver extract prepared from a crude liver concentrate by repeated treatment with fuller's earth. This, in addition to thiamine, riboflavin and nicotinic acid, was the only source of the vitamin B complex.

Street, Cowgill and Zimmerman (13) fed four dogs a basal diet supplemented with thiamine, riboflavin and filtrate factor concentrate and observed anorexia, intermittent diarrhea, abnormal gait and microcytic hypochromic anemia. The anemia could be relieved by the administration of a B_6 concentrate but crystalline "factor I" failed to influence the anemia even in doses of 500 μg. per kg. per day. Another unexpected finding was the development of symptoms of cardiac failure in three out of four animals. Only one dog was observed to have seizures suggestive of the epileptiform attacks described in pigs (8). Such attacks are not mentioned by Fouts et al. (11) or by Borson and Mettier (12) in the study of adult dogs.

McKibbin and his co-workers (14) confirmed the findings of others (12) that a plateau is reached following the hemopoietic response to the administration of pyridoxine and that an unidentified substance contained in liver is required to secure complete return of the blood to normal. They also made the significant observation that the plasma iron content progressively increases as the anemia becomes more severe and returns to normal promptly following administration of pyridoxine and in association with the initial immediate and rapid blood regeneration. The blood copper was also measured but was not found to follow a clear pattern. It seemed to rise slightly after treatment with pyridoxine.

In rats, lack of factor I is associated with impaired growth (15), a form of dermatitis termed "rat acrodynia" by György (16,17) and, according to certain investigators (18,19), epileptiform fits. An effect attributed to vitamin B_6 deficiency in the rat which has received little attention in other animals is an increase in the content of fatty acids in the liver (20). Halliday (20) found that by the administration of choline this could be relieved in part but not completely. Gavin and McHenry (21) could not confirm this finding, but Engel (22) showed recently that prolonged feeding of a diet deficient in pyridoxine is necessary to produce fatty livers.

TABLE 1

Data for blood changes, convulsions and autopsy findings in pigs given no crystalline supplement of pyridoxine

EXPERIMENT, PIG NO., AND SEX	AGE YEAST OMITTED[1]	CRYSTALLINE VITAMIN SUPPLEMENT GIVEN[2]	APPEARANCE OF: Significant anemia[3]	APPEARANCE OF: Convulsions	BLOOD CHANGES[4] Remarks	Day of expt.	RBC	Ht.	MCV	MCHC	Retics.	TERMINATION Day of expt.	TERMINATION Manner[5]	AUTOPSY FINDINGS: Bone marrow Hyperplasia	Bone marrow Hemosiderosis	Spleen (hemosiderosis) Pulp	Spleen (hemosiderosis) Capsule and trabeculae	Liver Hemosiderosis Kupffer cells	Liver Hemosiderosis Lobules	Liver Fat	C.N.S.[6]
	days		*days*	*days*			$\times 10^6$	*cc.*	$c\mu$	*per cent*	*per cent*										
					Average normal values		7.93	46	58	33	1.0										
VII, 6-64 M	88	TRNChP	28	0	Maximal Anemia	68	3.55	14	39	22	12.2										
					After pyridoxine	75	5.95	34	58	30	30.2 (2nd d.)										
					After pyridoxine	170	4.10	28	69	34	3.0	171	K	0	0	0	+	0	0	0	+
6-73 F	88	TRNChP	69	64	Maximal Anemia	107	5.15	20	40	29	2.4	110	D	+	++	+++	++++	+	0	+	+
6-76 F	88	TRNChP	42	87	Maximal Anemia	111	4.95	17	35	33	0.6	113	K	++	+++	++++	++++	+	+	+	++
IX, 7-28 F	38	TRNChPIp	41	43	Maximal Anemia	149	3.20	12	38	27	0.4	149	D	++	+	+++	0	0	0	0	+
7-29 F	34	TRNChPIp	41	37	Maximal Anemia	159	4.99	20	40	28	1.0	171	K	++	++	+++	+++	0	0	+	+
7-30 M	34	TRNChPIp	41	22	Maximal Anemia	50	6.35	31	49	29	0.2	50	D	++	++	++	+++	+	+	+	++
7-31 F	32	TRNChPIp	41	54	Maximal Anemia	68	6.70	23	34	28	0.6	70	D	++	++	+++	+++	0	0	+	+[7]
7-32 M	32	TRNChPIp	26	66	Maximal Anemia	95	6.13	19	31	29	4.8										
					After pyridoxine	114	8.30	30	36	31	15.6 (5th d.)										
					After pyridoxine	177	7.25	36	50	32	0.2	177	K	0	+	0	+	0	0	0	+++
7-33 M	32	TRNChPIp	26	65	Maximal Anemia	54	6.43	26	40	30	2.2	65	D	+	++	++	+++	+	+	+	+
VII, 6-56 M	90	TRNP	47	112	Maximal Anemia	131	3.65	16	43	24	2.4	139	K	++	+++	++++	++++	+	+	+	+
6-60 M	90	TRNP	19	64	Maximal Anemia	84	4.90	16	32	29	5.2										
					After pyridoxine	95	7.30	38	52	32	24.4 (4th d.)										
					After pyridoxine	151	5.00	29	59	34	2.4	151	K	0	++	+	+++	+	0	0	++++
6-71 M	88	TRNP	91	70	Maximal Anemia	91	7.60	32	43	35	1.2	99	D	+	+	++	0	+	0	+	±

TABLE 1—(*Concluded*)

IX, 7-18F	38	TRNChIp	107	128	Maximal Anemia	209	6.95	23	37	28	0.4		209	K	++	+++	++++	++++	+	+	0	++
7-19 F	38	TRNChIp	52	101	Maximal Anemia	143	2.28	9	39	27	1.8		146	D	++	+	+++	++	+	0	0	++
7-20 M	36	TRNChIp	24	0	Maximal Anemia	24	6.60	31	47	35	1.4		64	D	+	0	+	0	0	0	+	++
7-21 F	36	TRNChIp	0	0	Maximal Anemia	3	6.75	39	58	31	1.8		15	D	+	0	++	0	+	0	0	±
7-22 F	36	TRNChIp	45	62	Maximal Anemia	142	4.06	14	36	30	8.6		149	D	+	+	+++	+	+	0	0	+
7-23 M	34	TRNChIp	72	51	Maximal Anemia	114	5.85	22	39	29	0.8											
					After pyridoxine	171	4.89	29	59	22	4.6		171	D	+	++	++	+	0	0	±	+++
7-24 M	33	TRNChIp	30	51	Maximal Anemia	72	8.20	31	38	28	0.2		123	D	+	0	++	0	0	0	0	++
7-25 F	32	TRNChIp	17	142	Maximal Anemia	167	5.96	25	41	30	1.2											
					After pyridoxine	193	7.25	35	49	31	4.2		193	K	+	0	0	0	0	0	0	+++
VI, 6-34 M	109	TRN	0	0	Maximal Anemia	147	7.23	38	53	33	1.2		147	K	0	+	+++	0	+	0	+	++
6-45 M	110	TRN	175	163	Maximal Anemia	175	7.20	33	46	30	4.2		191	D	++	++	++++	+	+	0	+	+++
VII, 6-55 F	90	TRN	57	78	Maximal Anemia	97	4.90	20	40	33	0.2											
					After pyridoxine	114	6.20	31	50	31	5.8	(5th d.)										
					After pyridoxine	166	6.20	32	52	35	1.8		166	K	0	0	0	0	0	0	+	+++
6-68 F	87	TRN	44	0	Maximal Anemia	94	5.00	21	42	30	4.8											
					After pyridoxine	98	5.85	32	55	28	11.4	(4th d.)	104	D	+	+	+++	0	+	0	0	+++
6-69 M	88	TRN	86	0	Maximal Anemia	96	6.50	28	44	34	0.4											
					After pyridoxine	104	7.95	39	48	34	8.0	(5th d.)	110	D	+	0	++	0	0	0	+	+++

[1] Since this represents the time at which the crystalline vitamin supplements replaced yeast, this column indicates the age at which the experiment is regarded as having commenced. The time of development of anemia or convulsions, and other data which show "day of experiment" are computed from this day.

[2] T refers to thiamine; R, riboflavin; N, nicotinic acid; Ch, choline; P, pantothenic acid; I, inositol; p, para-amino benzoic acid.

[3] "Significant" anemia refers to a reduction in volume of packed red cells below 35 cc.

[4] Ht. refers to volume of packed red cells; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration. Under Retics. (reticulocytes), the day on which the maximal value was observed is indicated.

[5] K means sacrificed; D, died.

[6] The method of scoring the changes in the nervous system is described elsewhere (1, 6).

[7] Autolysis made evaluation of changes in the ganglion cells impossible.

MATERIAL AND METHODS

Full details of the experimental method have been given elsewhere (1, 6). The pigs, received at the laboratory at about three weeks of age, were fed the basal diet (Sheffield "New Process" casein, 26.1 per cent; sucrose, 57.7 per cent; lard, 11.0 per cent; salt mixture No. 3, 5.2 per cent) together with Mead's blended oil (supplying vitamins A and D) and brewers' yeast. After a certain length of time (see table I), yeast was withdrawn and was replaced by crystalline vitamins or by crude substances derived from liver. Studies of the nervous system and details concerning the growth of a number of the animals included in the present report have already been published elsewhere (1, 6, 7).

The quantities of crystalline substances, when these were given, (table I), were as follows: thiamine hydrochloride, 0.52, 0.26 or 0.13 mg. per kilogram body weight daily, as described in detail elsewhere (1, 2, 7); riboflavin, 0.12 mg. per kilogram body weight daily; nicotinic acid, 1.20 mg.; pyridoxine hydrochloride, 0.20 mg.; choline chloride, 10.0 mg.; calcium pantothenate, 0.50 mg.; inositol, 0.1 mg.; para-amino benzoic acid, 0.1 mg.

Although pyridoxine deficiency developed, no doubt, in a number of pigs given crude substances, such as certain fractions of liver (7), this report will deal particularly with pigs given only crystalline vitamins in addition to the basal diet and blended oil. In such animals the composition of the diet was better defined than in those receiving crude substances as the source of the vitamin B complex and the manifestations of deficiency could with more assurance be attributed to the vitamin which the diet lacked. Details concerning the nature of the crystalline vitamins given, the changes in the blood which took place and their response to treatment, the time of appearance of convulsions and the autopsy findings are given in table I.

OBSERVATIONS

Anemia. Anemia has been observed to develop in all the pigs given no supplement of pyridoxine. A significant degree of anemia, that is a volume of packed red cells of 35 cc. or less, has been noted as early as 4 weeks following substitution of crystalline vitamins (without pyridoxine) for yeast. Rarely anemia has appeared even in 3 weeks. In other animals anemia did not appear before 6 or even 10 or 15 weeks. The cause for this individual variation is not clear. Anemia developed not only in all pigs given no supplement of pyridoxine (9 pigs) but also in those given no supplements of other vitamins in addition to pyridoxine, such as choline (3 pigs), calcium pantothenate (8 pigs), or combinations of these (5 pigs). The same form of anemia was also observed in 14 out of 16 pigs fed liver fractions believed to contain little or no pyridoxine (7). The anemia to be described has not, on the other hand, ever been

seen in pigs fed crystalline pyridoxine in adequate amounts or in animals given crude substances such as liver or yeast which contain pyridoxine.

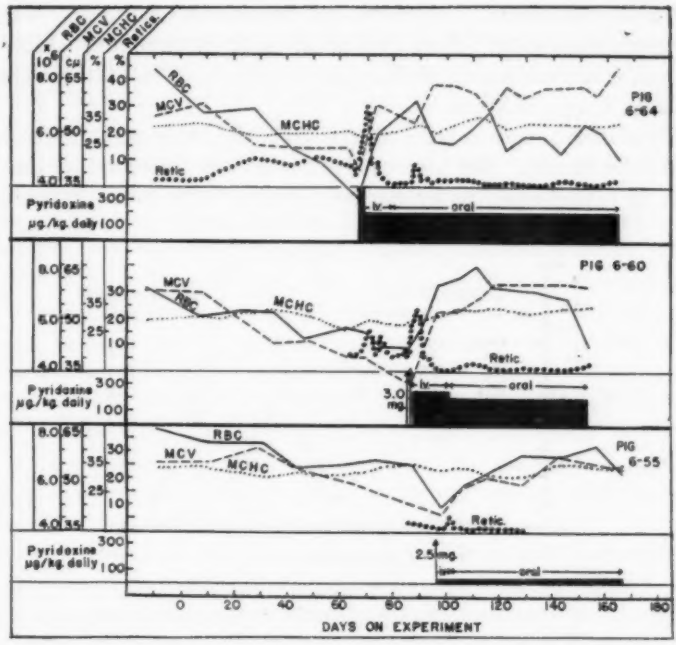


FIG. 1. DEVELOPMENT OF MICROCYTIC ANEMIA IN THREE PIGS DEFICIENT IN PYRIDOXINE AND RESPONSE TO TREATMENT

Note the promptness of the reticulocyte response and the rapid regeneration of red corpuscles of normal size. The anemia developing terminally in pig 6-60 was probably the result of bleeding due to prolapse of the rectum but no such cause was found for the somewhat macrocytic anemia which developed in pig 6–64 in spite of continued administration of pyridoxine.

Note also the less pronounced effect of treatment in pig 6-55 as compared with 6-64 and 6-60. Pig 6-55 received much smaller quantities of pyridoxine than the other two animals.

The most clearly defined picture has been seen in pigs given all the crystalline B vitamins excepting pyridoxine. Once significant anemia appeared it often progressed in the course of 2 to 4 weeks (figure 1, pig

6-64) to an extreme degree. Hemoglobin values as low as 4.0 and even 2.4 gm. per 100 cc. blood, and volume of packed red cells as low as 14.0 and even 9 cc. have been found in our pigs. The red cell count is not reduced proportionately and the anemia is strikingly microcytic. Values for mean corpuscular volume even as low as 32 c.μ. have been found and 40 c.μ. is not uncommon in this type of anemia. The average normal value in the pig is 58 c.μ.

Pyridoxine anemia is often referred to in the literature as microcytic and hypochromic. We have not been as greatly impressed by the degree of hypochromia as by the microcytosis. The mean corpuscular

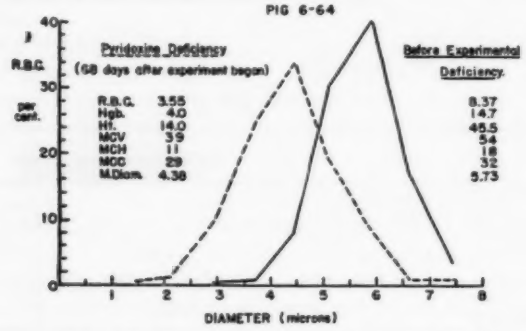


FIG. 2. ERYTHROCYTE DIAMETER FREQUENCY DISTRIBUTION ("PRICE-JONES") CURVES IN A PIG, SHOWING THE DEVELOPMENT OF MICROCYTOSIS WITH INCREASE OF ANISOCYTOSIS AS PYRIDOXINE DEFICIENCY BECAME PRONOUNCED

hemoglobin concentration in our animals was usually 28 to 33 per cent (normal 33 per cent), rarely lower. A number of the red corpuscles during the height of the anemia appear hypochromic but many are well filled with hemoglobin, some exceptionally so (plate I, figure 6).

As anemia develops anisocytosis becomes marked but poikilocytosis is rare. Large polychromatophilic red corpuscles make their appearance but at the same time microcytes become more and more plentiful so that the "Price Jones" curve of distribution of red cell diameters is "shifted to the left" and the mean diameter is reduced (figure 2). Some of the microcytes are extremely minute, measuring no more than 2 or 3 μ in diameter. An irregular reticulocytosis as great as 10 to 12 per cent may

appear (figure 1, pigs 6-64 and 6-60) and, in addition to the polychromatophilia already described, a number of red corpuscles are seen to contain a single, round and moderately large, blue-staining granule resembling a nuclear particle. Normoblasts, which may be found in the circulating blood of pigs in the absence of anemia, may become quite numerous when anemia becomes pronounced, as many as 4 and even 8 per 100 leukocytes having been seen in the blood of several of our animals.

We have observed no increase in the icterus index except in one animal at death. No significant alteration in the resistance of the red corpuscles to hemolysis in hypotonic saline solutions has been found. The corpuscles of the pig normally are much more sensitive to hypotonic saline solutions than are those of man, beginning hemolysis commencing in 0.72 to 0.66 per cent sodium chloride, well marked hemolysis is found at 0.63 to 0.54 per cent and complete hemolysis occurs at 0.48 to 0.36 per cent. In some anemic animals complete hemolysis did not take place until solutions of 0.33 or 0.30 per cent were used.

The leukocyte count in the pig fluctuates a great deal. We have been able to make out no distinct pattern in the total or differential leukocyte count in relation to the anemia although in a few instances, during the height of the anemia, a slight increase in the myeloid series of leukocytes occurred. Platelets appeared to be unaffected.

It will be noted in table I that the anemia developing in pigs not given calcium pantothenate or choline chloride as well as pyridoxine, was similar in all respects to that observed in pigs lacking only pyridoxine.

The immediate *effect of treatment* with pyridoxine was often striking. The percentage of reticulocytes increased sharply, reaching a peak on the second to the sixth day following administration of the first dose (figure 1). This was followed by rapid regeneration of blood, the increase affecting particularly the hemoglobin and volume of packed red cells as well as the size of the corpuscles.

The great rapidity with which these changes can take place is illustrated particularly by pigs 6-64 and 6-60 (figure 1) in which prompt increases in reticulocytes to 30 and 24 per cent, respectively, occurred while in a week the hemoglobin and volume of packed red cells were more than doubled. These changes were accompanied by a restoration of the mean corpuscular volume to normal, although it was evident from examination of the blood smears that great variation in the size of the cells

persisted for an additional two-week period. This extraordinarily rapid regeneration of blood accompanied the intravenous administration of pyridoxine in one dose of 0.4 mg. per kg. and subsequent daily doses of 0.2 mg. per kg. body weight in pig 6-64; and two injections of 3.0 mg. per kg. followed by 0.25 mg. per kg. daily in pig 6-60.

Treatment with pyridoxine was carried out in 7 other pigs (table I). In general, the magnitude of the changes in the blood appeared to be related to the size of the dose given, the degree of anemia and the route of administration. The highest reticulocyte increases and the greatest degrees of increase in hemoglobin and volume of packed red cells were observed when the anemia was most severe and large amounts of pyridoxine were given intravenously as already described in pigs 6-64 and 6-60 (figure 1 and table I). The observations in pigs 7-23 (figure 3) illustrate the effect of small quantities of pyridoxine given by mouth.

Employing the method of the "double reticulocyte response" (23) which has been used for assaying the potency of liver extracts in pernicious anemia, pyridoxine was given in doses of increasing size for periods of eight days or more. It will be noted that no change in the degree of anemia occurred when as little as 10 and 20 μg. pyridoxine per kilogram body weight were given daily *per os*. Very slight increases in reticulocytes appeared nevertheless on the sixth and fifth days, respectively, after administration of these amounts of pyridoxine was commenced. When calcium pantothenate was substituted for pyridoxine, the anemia became more severe. When 40 μg. pyridoxine was furnished daily a more substantial reticulocyte response developed than had been observed before and the anemia for the first time became less severe. An additional rise occurred when the dose of pyridoxine was increased to 80 μg. Further observations were interrupted by the death of the animal as the result of pneumonia. It is to be noted that this animal was deficient in pantothenic acid as well as pyridoxine and its gait was quite ataxic. The presence of secondary infections may have impaired the magnitude of the response of the blood to pyridoxine.

In pig 7-32 (figure 3) the oral administration of 20 μg. pyridoxine per kilogram body weight daily was followed by a well pronounced reticulocyte response and a rise in red cell count, hemoglobin and volume of packed red cells. Twice this amount of pyridoxine produced a somewhat further rise. The administration of 40 μg. per kilogram body weight

daily intravenously had a still more pronounced effect which, judging by the absence of a further change when still larger amounts were given, was probably maximal.

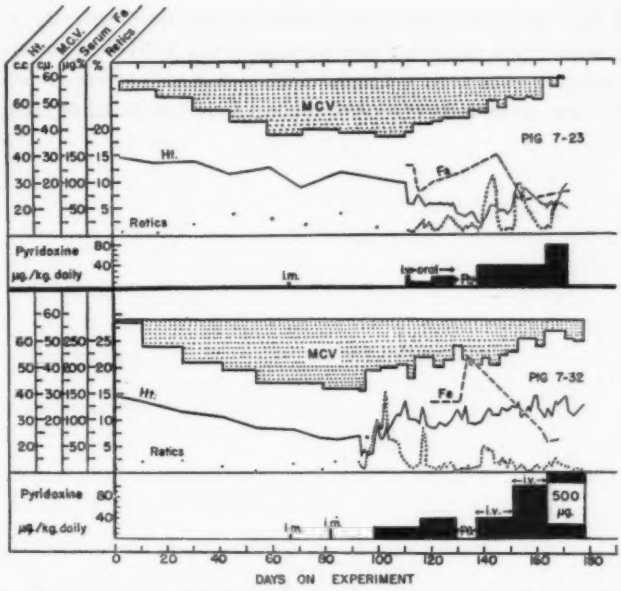


FIG. 3. CHANGES IN THE BLOOD IN TWO PIGS DEFICIENT IN PYRIDOXINE AND RESPONSE TO TREATMENT

Note (1) the increasing degree of microcytosis (M.C.V.) as anemia developed and the return towards normal as treatment was given; (2) the elevated serum iron (Fe) and the fall in relation to treatment; (3) the reticulocyte response to successively increased doses of pyridoxine and the lessening of anemia (Ht) as greater amounts were given; and (4) the increase of anemia and the rise in serum iron when pantothenic acid (Pa) was substituted for pyridoxine for 10 days.

Pyridoxine was given orally except where otherwise indicated (i. m. = intramuscularly; i. v. = intravenously).

Several investigators (12,14) have reported failure to relieve the anemia completely by the administration of pyridoxine. This was true also in the majority of our animals. As can be seen in figure 1, the blood values in pig 6-60 returned to normal after treatment with pyridoxine

and remained so except terminally when bleeding from a prolapsed rectum produced anemia. In pig 6-64, however, restoration of normal blood was only temporary and a somewhat macrocytic anemia gradually developed. Slight anemia began to appear also after a time in pig 6-55, and in the remaining pigs treated with pyridoxine completely normal values were never attained: two animals (6-68, 6-69) died too soon to allow restoration of normal blood values and in two others (7-23, 7-32) possibly the amounts of pyridoxine given were too small or secondary

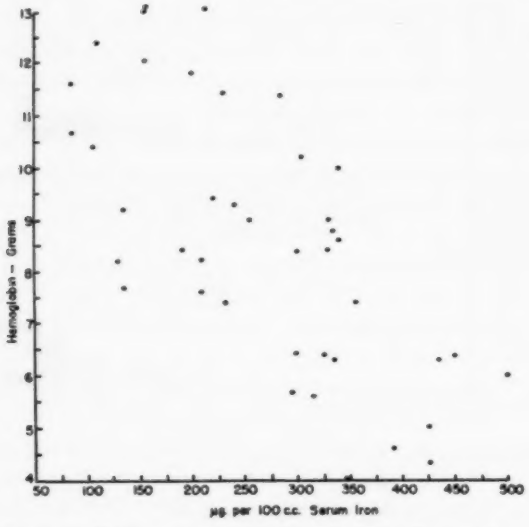


FIG. 4. TO SHOW THE TENDENCY FOR THE SERUM IRON TO RISE AS ANEMIA BECAME MORE SEVERE IN PYRIDOXINE DEFICIENT PIGS

infections inhibited the hematopoietic response. In pig 7-25 no such reasons for failure to restore the blood to normal were found.

It has been reported that the residual anemia can be corrected by the administration of liver extract (14). Liver was not given to the pigs included in the present report.

Measurements of *serum iron* were made in a number of the animals by the method of McKibbin et al. (14). As has been reported by others (11, 14) we found that the serum contained abnormally large quantities

of iron. While irregularities were noted, the iron tended to rise as the hemoglobin fell (figure 4). Treatment was followed by a return of the serum iron to normal values (table II).

Histologic findings related to the anemia. Well pronounced and uniform changes were found in the spleen, liver and bone marrow of the animals

TABLE II

Serum iron determinations in pyridoxine deficient pigs

FIG. NO.	TYPE OF DEFICIENCY	AVERAGE SERUM IRON
		mg. per 100 cc.
7–28	Pyridoxine	214
7–29	Pyridoxine	362
7–32	Pyridoxine	220
	After treatment with pyridoxine	60
7–17	Pyridoxine and pantothenic acid	236
	After treatment with pyridoxine	108
7–18	Pyridoxine and pantothenic acid	318
	After treatment with pantothenic acid	276
7–19	Pyridoxine and pantothenic acid	394
7–22	Pyridoxine and pantothenic acid	238
	After treatment with pantothenic acid	226
7–23	Pyridoxine and pantothenic acid	301
	After treatment with pyridoxine and pantothenic acid	99
7–25	Pyridoxine and pantothenic acid	208
	After treatment with pyridoxine and pantothenic acid	111

developing pyridoxine anemia. Since certain pigs died or were sacrificed after the anemia had been successfully relieved by the administration of pyridoxine whereas others died at the time they were anemic, it was possible to observe the effects produced by therapy as well as the histologic changes associated with the anemia.

The changes related to the presence of anemia were hemosiderosis in

the spleen, liver and bone marrow and hyperplasia of the bone marrow. In the spleens there was a great accumulation of hemosiderin in the pulp where it was found in cells and as extracellular clumps as well, but it was conspicuously absent from the Malpighian bodies (plate II, figure 10). Even when anemia was only moderate in degree (pigs 7-30, 7-33, 6-71, 7-20, 7-21, 7-24, 6-34, 6-45, table I) the deposition of pigment in the pulp was well marked and sometimes even extensive. About the edges of the trabeculae and the inner border of the capsule pigment was more often found where the anemia was severe (pigs 6-76, 6-73, 6-56, table I). Pigment was also found in the Kupffer cells of the liver and, when the anemia was most severe, in the periportal cells of the liver lobules (plate II, figure 12), and was present in large quantities in macrophages of the bone marrow. No pigment was found in the renal tubular epithelium except in pig 7-19. It is noteworthy that this pig was more anemic than any other animal.

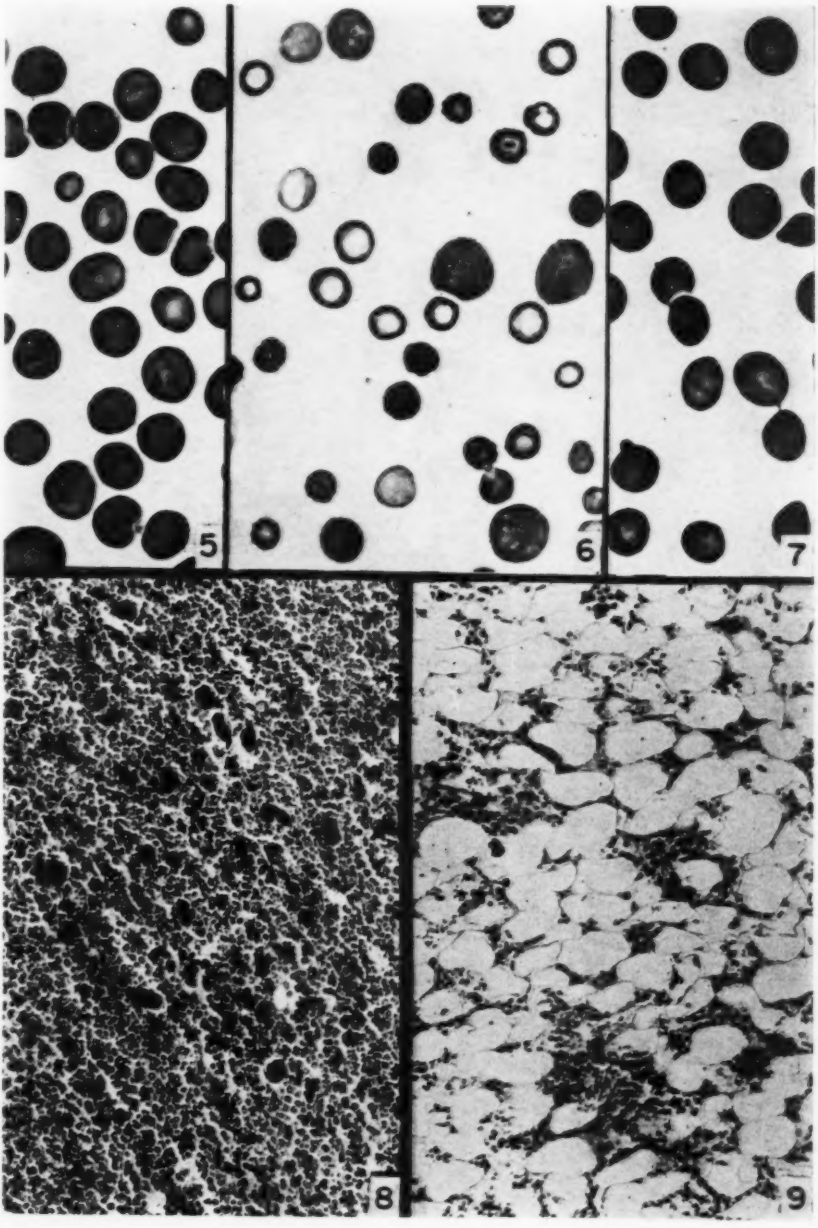
The effect of treatment with pyridoxine appeared to be to diminish the amount of pigment in the splenic pulp. The liver cells and macrophages in the bone marrow also showed no pigment in animals which had been treated. The duration of therapy could be definitely correlated with the amount of pigment remaining in the pulp of the spleen, but that in the capsule and trabeculae was to a large extent retained even after prolonged treatment (pig 6-64, table I and plate II, figure 11). This seemed to indicate that the iron for red blood cell formation was much more easily mobilized from the pulp than from the capsule and trabeculae.

In marked contrast to the findings in animals in which pyridoxine

PLATE I

FIGURES 5–7. Red corpuscles of a pig (6-64) (5) at the start of the experiment when the blood was normal; (6) 77 days later after severe pyridoxine-deficiency anemia had developed; and (7) 28 days later, following treatment with pyridoxine as shown in figure 1. Erythrocyte diameter measurements of the red blood cells illustrated in figures 5 and 6 have been presented in figure 2. Photomicrographs. Wright's stain, × 1200.

FIGURES 8 AND 9. Femur bone marrow to show (8) the extensive hyperplasia in a pig (6-56) sacrificed when anemia was severe; and (9) the relatively fatty marrow of a pig (7-32) originally anemic but subsequently treated with pyridoxine. Prussian blue fuchsin stain, × 150.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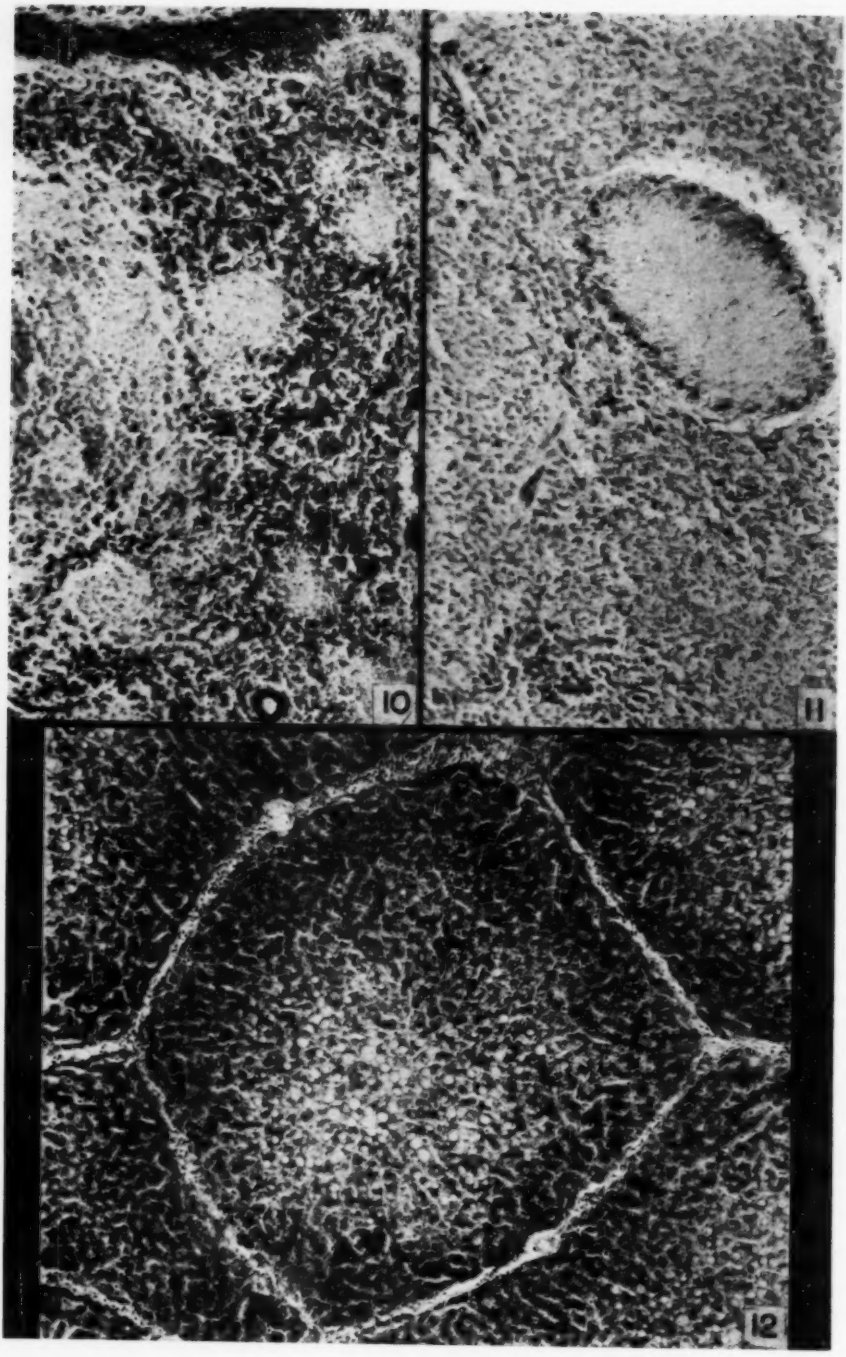
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anemia had been present, virtually no pigment was found in the tissues of control animals receiving adequate pyridoxine from the beginning of the experiment.

The bone marrow of the pyridoxine deficient animals was hyperplastic in comparison to that of the controls. The marrow removed from the femur was cellular in the former group while in the latter animals it was fatty. The cellularity was due to an increase in clearly discernible nucleated red blood cells as well as in undifferentiated "blast" cells. In animals which had received adequate treatment, little or no bone marrow hyperplasia was found (plate I, figure 9).

Convulsions. Convulsions have been observed in 20 out of 26 pigs not given a supplement of pyridoxine as well as in 6 out of 16 pigs fed fractions of liver poor in pyridoxine. Since the animals were not under constant surveillance, failure to record convulsions in some of the pigs may have been due to the fact that they occurred at night or at certain periods during the day when they might not have been noted. Convulsions have been observed as early as 3 weeks following commencement of the experimental deficiency but most often they have been first noted 7 to 12 weeks after the start of the experiment. We have observed no consistent relationship between the time of onset of convulsions and the time at which significant anemia has appeared, although in about two-thirds of the animals convulsions have been noted for the first time 3 to 7 or even 15 weeks after such anemia first developed.

Since we have already described the "fits" occurring in the pigs (7), they need little further description here. In pronounced cases they re-

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PLATE II

FIGURES 10 AND 11. Photomicrographs of sections of spleens of (10) a pig (6-76) sacrificed when anemia was severe. Note the extensive deposition of iron pigment in the trabecula (at top) and in the pulp. The areas free of iron pigment are Malpighian bodies; and (11) a pig (6-64) which had been very anemic but was treated with pyridoxine (see figures 1, 2, 5, 6, 7). Note that there is virtually no iron pigment except for traces in the periphery of the trabecula shown in cross-section in the middle of the field and in longitudinal section in the upper left corner of the photomicrograph. Prussian blue fuchsin stain, × 150.

FIGURE 12. Liver of a pig (6-76) deficient in pyridoxine, to show deposition of fat in the central portion of the lobule. Note also the deposits of iron pigment in cells in the peripheral portion of the lobule. Prussian blue fuchsin stain, × 100.

semble those seen in the "grand mal" of human epilepsy. "Petit mal" attacks of shorter duration than the more severe episodes and not ending in coma have also been observed. In such instances the pig may have several jerky movements of the whole body or may bounce backwards suddenly. In a somewhat more marked form, these jerky movements are more pronounced and longer continued, the pig may hold its head stiffly erect and the limbs stiffly extended so that the jerky movements of the animals strutting about the pen resemble that of a mechanical toy which has been wound up. Following these minor episodes which may last from a few seconds to one or two minutes the animal may walk or stagger about in a dazed manner but does not pass into coma.

Once convulsions have made their appearance in an animal they may be repeated several times in a day and death may occur following one of them, or they may occur infrequently at unpredictable intervals. Animals subject to convulsions seem irritable and "nervous." Excitement has seemed often to have been a precipitating factor in bringing on a convulsion. Their behaviour as compared with that of pigs not deficient in pyridoxine is less purposeful. The eyes often are not held open as widely and sometimes the animals may appear to have photophobia or may behave temporarily as if blind.

Even though convulsions have occurred at irregular intervals, it has been evident that convulsions cease promptly following administration of pyridoxine in the doses used in the treatment of the anemia. From our observations to date it would appear that a single intramuscular injection of 20 μg. pyridoxine per kg. body weight may cause a temporary cessation in the occurrence of convulsions. This is probably the minimal amount. Half this quantity definitely has no beneficial effect. The *daily* oral administration of 20 μg. and even 10 μg. per kg. body weight appeared to have put an end to the occurrence of convulsions in one pig (7-23) but these no doubt are minimal amounts for 20 μg. was not sufficient in the case of pig 7-32. In the latter no more convulsions were observed after 40 μg. was given daily. We have no adequate data regarding the effects of pyridoxine administered during a convulsion.

No changes in the brain have been found in any of the pyridoxine deficient animals, except in the lower medulla. Here there were fibers in the ascending sensory tracts that had lost their myelin sheaths.

These changes were interpreted as being part of the sensory neuron degeneration we have described as resulting from pyridoxine deficiency (1).

Other manifestations of pyridoxine deficiency. There is distinct impairment of growth in pigs given no pyridoxine although this effect is by no means as striking as is found in association with pantothenic acid deficiency (1). Some animals may appear only moderately thin and the hair may be quite tidy and clean. Others, particularly those which have survived a longer time, may show more evidence of failure to gain weight, the hair may become somewhat untidy and curled, or diarrhea may develop with consequent marked deterioration in the appearance of the animal. If the pig survives long enough to develop marked incoordination of gait its appearance deteriorates even more.

In the urine of certain pyridoxine deficient pigs we have been able to demonstrate the green pigment developing on the addition of ferric ammonium sulfate which Lepkovsky and Nielsen described recently (24).

Pathological changes in the liver. Besides the hemosiderosis of the spleen, liver and bone marrow, the hyperplasia of the bone marrow and the degenerative changes in the sensory neuron which we have discussed already, the only pathological findings observed in our pyridoxine deficient animals which have appeared with regularity have been in the liver. These consisted of fatty infiltration and could be seen as vacuoles in the cells of the central portions of the liver lobules. In some cases of severe deficiency fat was found in the mid-zonal regions as well. As will be seen in table I fatty infiltration was present in animals deficient in pyridoxine in all but six instances. Five of these six animals were also deficient in pantothenic acid and one of the five lived only 15 days on the deficient diet. It is noteworthy that the fatty infiltration occurred even when choline was present in the diet and when inositol and para-aminobenzoic acid were also added.

Of eight pigs originally deficient but examined following administration of pyridoxine, the liver was normal in six and showed fat infiltration in two. These two animals had not received supplements of choline or pantothenic acid. The finding of fatty livers in these two pigs could therefore be attributed to the absence of choline as a dietary supplement. Pig 6-60, which had also received no supplement of

choline during the developmental stage of the deficiency, was given choline as well as pyridoxine when treatment was commenced. No fatty infiltration was found at autopsy. The findings in pig 6-68, however, were at variance with these results for even though no choline supplement had been given no excess of fat was found in the liver. This pig, it should be noted, had also received no supplement of pantothenic acid.

We have pointed out elsewhere (1) that lack of pantothenic acid appears to counteract the effect of choline and pyridoxine deficiency on the deposition of fat in the liver. The observations recorded here are consistent with this conclusion and, with only one exception (pig 7-28) also indicate that fatty infiltration of the liver occurs when either pyridoxine or choline, or both are not furnished as supplements to the diet. The presence or absence of inositol as a supplement appeared to have no relation to the findings in the liver, but it is to be noted that the amounts of inositol given were rather small. The presence or absence of fat in the livers of pyridoxine-deficient pigs did not seem to be correlated with the degree of anemia that existed.

DISCUSSION

It is evident that in the pig, as in the dog, pyridoxine plays an important role in blood formation. This role appears to be related in some way to iron metabolism. The plasma iron increases as pyridoxine anemia develops and marked hemosiderosis of the tissues occurs at the same time. There appears to be a failure to utilize iron rather than a lack of it. The bone marrow is hyperplastic and yet is unable to produce normal red corpuscles. The rapidity of red cell formation once pyridoxine is administered suggests that this vitamin is concerned in some way in the utilization of iron in hemoglobin production. With effective treatment the serum iron falls, hemosiderosis disappears and there is rapid regeneration of red corpuscles of normal size and hemoglobin content.

There are a number of features in common between pyridoxine anemia and the nutritional anemias occurring in man; namely, pernicious anemia and iron deficiency anemia. In all three types of anemia the bone marrow is hyperplastic and the rapidity of blood regeneration which follows the administration of the substance which is

lacking, whether it be the antianemic principle in liver, or iron or pyridoxine, suggests that a break has been present in the normal metabolic chain of red blood cell formation. The changes occurring in a particular deficiency depend on the site at which the chain is broken. In pernicious anemia, as in pyridoxine anemia there is hemosiderosis in the spleen, liver and bone marrow and the plasma iron is elevated above normal. The lack of the antianemic principle of liver, however, causes the formation in the main of red corpuscles which are larger than normal. In iron deficiency anemia the red corpuscles possess morphologic characteristics similar to those of the cells in pyridoxine anemia but the serum iron is very low and the tissues are wanting in iron. Pyridoxine anemia possesses some of the features of both of these types of anemia; namely, microcytosis and hypochromia, yet high serum iron and hemosiderosis. The hemosiderosis, it should be noted, is not entirely like that of pernicious anemia for pigment is rare in the kidneys.

There is no evidence at this time that pyridoxine anemia occurs in man. "Mediterranean anemia" is a form of anemia in man which has certain features in common with pyridoxine anemia. The red corpuscles are microcytic and hypochromic and yet the anemia fails to respond to iron, and iron-containing pigment is found in certain tissues (23). Nevertheless, the administration of pyridoxine has been of no avail in influencing "Mediterranean" anemia.

The green pigment-producing compound (24) found in the urine in pyridoxine anemia may possibly be a substance related to blood formation which cannot be used when pyridoxine is lacking. There is no more justification for regarding pyridoxine anemia as fundamentally a "hemolytic" anemia than in the case of pernicious anemia. In the latter disorder bilirubinemia occurs and urobilinogen is found in the urine but the fundamental fault apparently is the lack of a specific, as yet unidentified blood-building substance. Our own investigation of the excretion of urobilin in the stools and urine of pyridoxine-deficient animals has so far shown no consistent trend.

Judging by what is known about certain other vitamins, it is plausible to assume that pyridoxine is necessary for the formation of an enzyme concerned in erythropoiesis. It is also worth noting that pyridoxine resembles copper and cobalt in that it appears to be con-

cerned with iron in the process of blood formation. Copper is thought to facilitate or to be essential for the utilization of iron by the blood-forming organs and for the mobilization of iron from the tissues (25). As to cobalt, the tissues of sheep suffering from cobalt deficiency have been found to contain abundant amounts of iron (25).

It is very probable that the epileptiform convulsions seen in pyridoxine-deficient animals are also caused by a metabolic disturbance. Our own preliminary studies of the changes which occur during the seizures have shown a fall in the pH and a decrease in the CO_2 of the venous blood, both of which varied in degree with the severity and duration of the episodes. The hydrogen iron concentration of the blood has been found reduced in several instances even in the absence of convulsions. We have noted no significant alterations in the plasma chlorides, total protein or calcium. The blood sugar has been found markedly increased in several instances and a moderate increase in non-protein nitrogen has also been noted. Some of these changes can be accounted for by the muscular activity accompanying the convulsive seizures.

The occurrence of fatty infiltration of the liver in the pyridoxine deficient pigs confirms the observations of Halliday (20) and Engel (22) in rats. As already stated, however, fatty livers were observed in our animals whether inositol was given or not. This failure to demonstrate a need for inositol is not necessarily significant for the amounts fed possibly were insufficient. Furthermore, histological methods are much less accurate than chemical determinations of fat content as carried out by Engel (22).

The absence of fatty infiltration of the liver in a number of pigs deficient in pyridoxine or in choline when pantothenic acid was also lacking, does not necessarily indicate a direct antagonistic role on the part of the last named vitamin. In the pig, growth is more impaired by lack of pantothenic acid than in deficiencies of any other vitamins (1). The decreased need for pyridoxine and choline as lipotropic factors may possibly be due to the very low food consumption in pantothenic acid-deficient pigs.

SUMMARY

1. Pyridoxine deficiency leads to the development of a severe anemia in pigs. This is characterized by microcytosis, an increase of

polychromatophilia, reticulocytes and nucleated red cells in the blood, a rise in the serum iron, bone marrow hyperplasia and hemosiderosis in the spleen, liver and bone marrow.

2. Administration of pyridoxine is followed by a sharp reticulocyte response and rapid regeneration of blood with restoration of the normal size of the red corpuscles. Mobilization of iron from the tissues and its utilization in blood formation is indicated by the disappearance of hemosiderosis and a fall in the serum iron. The intensity of the response appears to be related to the amount of pyridoxine given and, probably, the route of administration.

3. Pyridoxine deficiency leads also to the development of seizures in pigs which somewhat resemble the "petit mal" and "grand mal" of human epilepsy. These do not recur if adequate quantities of pyridoxine are given.

4. Prolonged feeding of a diet deficient in pyridoxine is associated with the development of fatty infiltration of the liver.

5. It is suggested that pyridoxine deficiency leads to the development of a metabolic disorder which, insofar as the anemia is concerned, is related to the utilization of iron. The nature of the mechanism by which pyridoxine deficiency arrests hemoglobin synthesis and elevates serum iron is not understood.

Vitamins used in these experiments were furnished by Merck and Company, Incorporated, and yeast and cod liver oil by Mead Johnson and Company.

Technical aid was given by Mrs. Lottie Lowenstein, Mrs. Eleanor Collins, Miss Eva Hodgens, Miss Elaine Cohen and Miss Gertrude Merr.

BIBLIOGRAPHY

(1) Wintrobe, M. M., Miller, M. H., Follis, R. H., Jr., Stein, H. J., Mushatt, C., and Humphreys, S.: Sensory neuron degeneration in pigs. IV. Protection afforded by calcium pantothenate and pyridoxine. *J. Nutrition*, **24**: 345, 1942.

(2) Wintrobe, M. M., Stein, H. J., Miller, M. H., Follis, R. H., Jr., Najjar, V., and Humphreys, S.: A study of thiamine deficiency in swine, together with a comparison of methods of assay. *Bull. Johns Hopkins Hosp.*, **71**: 141, 1942.

(3) Follis, R. H., Jr., Miller, M. H., Wintrobe, M. M., and Stein, H. J.: Development of myocardial necrosis and absence of nerve degeneration in thiamine deficiency in pigs. *Am. J. Path.* (to be published March, 1943, vol. 19).

(4) WINTROBE, M. M., MILLER, M. H., FOLLIS, R. H., JR., AND STEIN, H. J.: What is the antineuritic vitamin? *Tr. Ass. Am. Phys.*, **57:** 55, 1942.

(5) WINTROBE, M. M., MITCHELL, D. M., AND KOLB, L. C.: Sensory neuron degeneration in vitamin deficiency. *J. Exper. Med.*, **68:** 207, 1938.

(6) WINTROBE, M. M., MILLER, J. L., JR., AND LISCO, H.: The relation of diet to the occurrence of ataxia and degeneration in the nervous system of pigs. *Bull. Johns Hopkins Hosp.*, **67:** 377, 1940.

(7) WINTROBE, M. M., MUSHATT, C., MILLER, J. L., JR., KOLB, L. C., STEIN, H. J., AND LISCO, H.: The prevention of sensory neuron degeneration in the pig, with special reference to the role of various liver fractions. *J. Clin. Investigation*, **21:** 71, 1942.

(8) CHICK, H., MACRAE, T. F., MARTIN, A. J. P., AND MARTIN, C. J.: The water-soluble B-vitamins other than aneurin (vitamin B_1), riboflavin and nicotinic acid required by the pig, *Biochem. J.*, **32:** 2207, 1938.

(9) WINTROBE, M. M., SAMTER, M., AND LISCO, H.: Morphologic changes in the blood of pigs associated with deficiency of water-soluble vitamins and other substances contained in yeast. *Bull. Johns Hopkins Hosp.*, **64:** 399, 1939.

(10) FOUTS, P. J., HELMER, O. M., LEPKOVSKY, S., AND JUKES, T. H.: Production of microcytic hypochromic anemia in puppies on synthetic diet deficient in rat antidermatitis factor (vitamin B_6). *J. Nutrition*, **16:** 197, 1938.

(11) FOUTS, P. J., HELMER, O. M., AND LEPKOVSKY, S.: Nutritional microcytic hypochromic anemia in dogs cured with cystalline factor I. *Am. J. M. Sc.*, **199:** 163, 1940.

(12) BORSON, H. J., AND METTIER, S. R.: Relief of hypochromic anemia in dogs with synthetic vitamin B_6: Influence of "filtrate factors." *Proc. Soc. Exper. Biol. & Med.*, **43:** 429, 1940.

(13) STREET, H. R., COWGILL, G. R., AND ZIMMERMAN, H. M.: Some observations of vitamin B_6 deficiency in the dog. *J. Nutrition*, **21:** 275, 1941.

(14) MCKIBBIN, J. M., SCHAEFER, A. E., FROST, D. V., AND ELVEHJEM, C. A.: Studies on anemia in dogs due to pyridoxine deficiency. *J. Biol. Chem.*, **142:** 77, 1942.

(15) DIMICK, M. K., AND SCHREFFLER, C. B.: The factor I (vitamin B_6) requirement of the rat. *J. Nutrition*, **17:** 23, 1939.

(16) GYÖRGY, P.: Investigations on vitamin B_2 complex; differentiation of lactoflavin and "rat antipellagra" factor. *Biochem. J.*, **29:** 741, 1935.

(17) ANTOPOL, W., AND UNNA, K.: Pathology of B_6 deficiency in the rat and response to treatment with 2-methyl-3-hydroxy 4,5-dihydroxymethylpyridine (vitamin B_6). *Proc. Soc. Exper. Biol. & Med.*, **42:** 126, 1939.

(18) CHICK, H., EL SADR, M. M., AND WORDEN, A. N.: Occurrence of fits of an epileptiform nature in rats maintained for long periods on a diet deprived of vitamin B_6. *Biochem. J.*, **34:** 595, 1940.

(19) DANIEL, E. P., KLINE, O. L., AND TOLLE, C. D.: A convulsive syndrome in young rats associated with pyridoxine deficiency. *J. Nutrition*, **23:** 205, 1942.

(20) HALLIDAY, N.: Fatty livers in vitamin B_6 deficient rats. *J. Nutrition*, **16:** 285, 1938.

(21) GAVIN, G., AND MCHENRY, E. W.: The B vitamins and fat metabolism. III. The effects of vitamin B_6 upon liver and body fat. *J. Biol. Chem.*, **132:** 41, 1940.

(22) ENGEL, R. W.: The relation of B-vitamins and dietary fat to the lipotropic action of choline. *J. Nutrition*, **24:** 175, 1942.

(23) WINTROBE, M. M.: Clinical Hematology. Lea and Febiger, Philadelphia, 1942.

(24) LEPKOVSKY, S., AND NIELSEN, E.: A green pigment-producing compound in urine of pyridoxine-deficient rats. *J. Biol. Chem.*, **144:** 135, 1942.

(25) SCHULTZE, M. O.: Metallic elements and blood formation. *Physiol. Rev.*, **20:** 37, 1940.

HORMONE PRODUCTION BY PLACENTAL CELLS MAINTAINED IN CONTINUOUS CULTURE[1]

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Since Aschheim and Zondek, in 1926, first demonstrated the presence of a gonadotropic hormone in the urine of pregnant women a wealth of evidence has accumulated which indicates that this substance is elaborated by the placental cells and not by the anterior pituitary as was originally believed.

Human chorionic gonadotropin has been shown to differ biologically from the luteinizing factor of the anterior hypophysis, LH, in that the chorionic hormone will produce growth of follicles in an immature, non-hypophysectomized rat. The two hormones also differ in their physical and chemical properties. The chorionic gonadotropin is more soluble in water than the pituitary hormone and apparently has no isoelectric point. Unlike the luteinizing hormone it is soluble and stable in tricresol. The response of the two hormones to enzymatic digestion also varies. The carbohydrate component of the pituitary hormone has been found to be mannose while that of the placental hormone is galactose. On the basis of these apparent differences, Astwood and Greep have proposed that the general term cyonin be used in referring to the gonadotropic substances of supposed placental origin in order to differentiate them from the luteinizing hormone, LH, of the anterior pituitary. This term will be used throughout the paper.

The evidence presented for a dual origin of these hormones, although very convincing, is however largely circumstantial and direct proof has been difficult to obtain. It seemed that such direct proof might be afforded by a study of placental cells growing in tissue culture, by determining whether the placental cells would elaborate hormones in vitro. Therefore pure strains of cells from human placental tissue were

[1] Presented in large part before the American Physiological Society on March 15, 1940, at New Orleans, La.

maintained in continuous culture. Many valuable fresh specimens were sent to us from the Boston Lying-In Hospital through the courtesy of Dr. A. T. Hertig and his colleagues. Assays for cyonin and estrogen were performed on the supernatant cell free culture medium, removed after having been in contact with growing placental cells for 3 or 4 days.

CULTURE METHODS

The culture technique employed was that described by Gey. The culture medium was balanced salt solution 10 per cent, beef embryo extract 10 per cent, human cord serum 40 per cent, and chicken plasma 40 per cent. In several instances human plasma obtained from pregnant and normal cyclic women was used in place of chicken plasma. However, no striking differences were observed in the growth of cultures so treated when compared with the control cultures maintained in chicken plasma. Tissue for assay was explanted into roller tubes which were rotated at a constant speed of 12 revolutions per hour. The supernatant fluid was changed every three or four days and the cultures were patched and transferred as necessary. For purposes of microscopic observation two types of cover slip preparations were used: (1) lying drop cultures on large cover slips (40–45 mm.), and (2) tube slip cultures made by inserting a ¼ x 1 inch No. 1 thickness cover slip on which cell colonies were explanted directly into a roller tube.

ASSAY METHODS

Specimens cultured were 5 placentae obtained by hysterotomy on patients who were three or four months pregnant, 2 placentae of ectopic pregnancies, 2 full term placentae, and 3 hydatidiform moles. Assays were performed on the cell free supernatant fluid which was removed after having been in contact with the living cultures for three or four days. Assays were carried out only when the tissues had been cultured at least two weeks, and when the culture tubes had had several changes of fluid media. By this time the tissue cultures no longer represented primary explants but were made up of several generations of new cells. The cyonin was assayed by the original Aschheim-Zondek technique, using, however, 21 day old rats instead of mice. In each case the ovaries were sectioned and examined microscopically for corpora lutea. Estrogen assays were performed by both the vaginal estrus and the six hour Astwood methods.

CYTOLOGICAL OBSERVATIONS

In general the microscopic observations regarding the type of cellular growth obtained from cultured placental tissue were in accord with those made by previous investigators (Guggisburg and Neuweiler, Heim, Friedheim, Segupta, Madruzza, Sannicandro, and Taszkan).

Usually there was a lag period of several days before migration occurred and no appreciable increase in cellular elements was observed

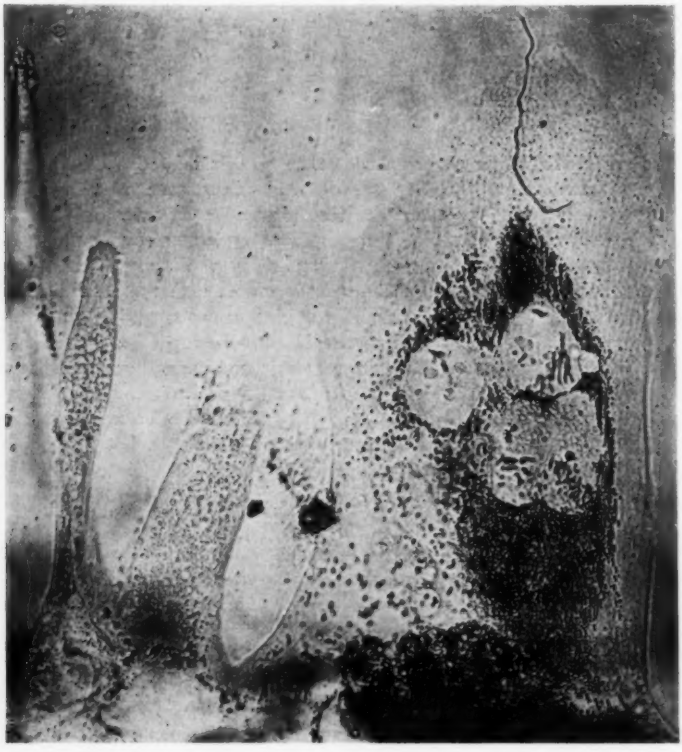

FIG. 1. High power photomicrograph of living cells from an hydatidiform mole growing in tissue culture. Typical blunt, villus-like processes can be seen pushing their way out from the edge of the culture. A multinucleated cell can be seen with numerous refractile granules. M. Mi. S-38-369 (Courtesy Dr. A. T. Hertig, Boston Lying-In Hospital). Explanted 27.IV.38. Culture Slide #1 photographed 13.V.38 (× 500).

until the cultures had been incubated from five to eight days. In the primary explants growth and migration of cells was observed mainly at the bases of the chorionic villi where fairly dense masses of the tropho-

blastic cells had been cut across. Very occasionally a limited migration of the syncytial cells was observed in the form of short, blunt villus-like processes, consisting of only a few cells. These syncytial processes were especially numerous in cultures of hydatidiform mole (fig. 1). In the latter, sheet-like outgrowths of syncytium were also observed. Active blunt villus-like processes are rarely seen in cultures of normal placentae. We have observed such active living

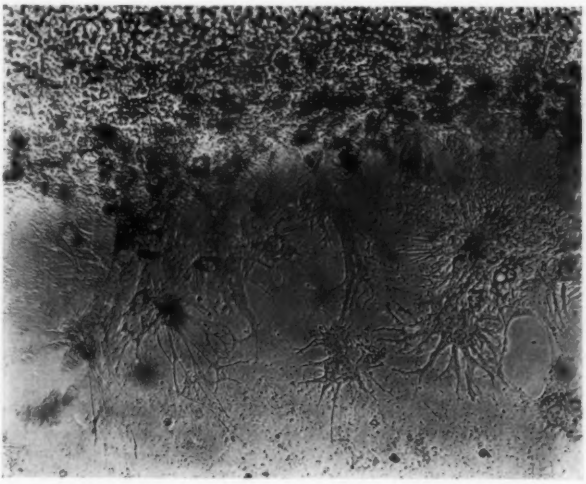

FIG. 2. Low power photomicrograph of a strain of placental cells from a malignant hydatidiform mole. Most of these cells have many very narrow long processes which give the cells a "burr-like" appearance. This is evidently comparable to the "brush" border seen in stained sections. Lo. LeCl. S-39-1144 (Courtesy Dr. A. T. Hertig, Boston Lying-In Hospital). Explanted. 6.XII.39. Roller Tube #2 photographed 19.XII.39 (× 150).

villus processes in cultures of full term placentae prepared shortly after Caesarian operation. The myxomatous stroma occasionally grew in the primary explants, but in most cultures it was eventually overgrown by the epithelial elements.

When definite tissue culture colonies were formed from placental tissue explants, these could be maintained in continuous culture as a uniform strain and the cells were usually of one general type. On

the basis of observations on the primary cultures these cells have been identified as those coming from the Langhans' layer of the chorionic villus. The type of cell growth observed was not the typical pavement sheet-like arrangement seen in most epithelial cultures but

FIG. 3A FIG. 3B

FIG. 3A. Low power photomicrograph of living placental cells from an ectopic pregnancy of the fourth month. This photograph represents a typical peripheral zone as found in cultures which can be maintained indefinitely. Many of the cells are distended with large clear refractile vacuoles. Ev. Gw. J. H. H. Gyn. Path. 46875. Explanted 17.VI.38. Maintained in continuous cultures 298 days when photographed. Roller Tube #81 photographed 11.IV.39. (× 200).

FIG. 3B. Low power photomicrograph of living placental cells from an ectopic pregnancy of the fourth month. Again the typical broad, elongated cells are illustrated. The one at the bottom is multinucleated. The cytoplasm of the central cell is almost entirely replaced by two large, clear vacuoles. The brush-like border of the bottom cell can be faintly discerned. Same culture tube as Fig. 3A. × 200).

rather a more radiating type of outgrowth. This outgrowth was made up of broad and elongate cells, many of them only loosely "connected" in areas by multiple long narrow processes (fig. 2). These evidently correspond to the "brush border" mentioned by Grosser in Keibal and Mall's text.

The individual cellular characteristics varied to some extent with the location of the cell in the colonies. In some cultures the cells were spread out very thinly at the periphery and there were numerous large, clear vacuoles (fig. 3) and highly refractile granules (fig. 1). Comparable vacuolar spaces could be seen in stained sections of normal uncultured placenta. The significance of these vacuoles in the cellular physiology is as yet undetermined. These were such a constant constituent of the cells that they probably have some functional significance. The cell nuclei were round or slightly oval, clear and more difficult to outline. Usually they contained one or more nucleoli. On many of the cells there were countless fine sharp pseudopodia which gave them a brush-like appearance. Mitoses were rare and occasionally multinucleated cells were seen. This may indicate the ability of these cells to form syncytial masses (fig. 1 and fig. 3B).

RESULTS

Twenty of twenty-nine specimens of fluid media from placental cultures when assayed for cyonin gave positive results as judged by the microscopic appearance of the immature rat ovary. Of these 20 assays, 14 showed corpora lutea formation and 6 showed follicle stimulation. Eight assays were negative and one showed questionable follicle stimulation (table 1).

Several interesting facts can be seen from a survey of table 1. The placental cells do not lose their ability to produce cyonin even after long periods in continuous culture. One assay, No. 1808, performed on fluid from tissue cultured for 6 months continued to show a positive result. However, it is apparent, from an examination of those specimens upon which repeated assays were obtained, that the cells tend to lose their ability to produce the hormone after having been in culture over long periods. From a cultural viewpoint, the growth of term placentae is of special interest as former investigators have claimed that these cannot be grown in tissue culture. The two series of cultures from full term placentae in the present series grew readily, and one gave positive tests for cyonin repeatedly. All of the types of placental specimens which were cultured gave positive cyonin assays. One series of decidual cultures gave negative results.

TABLE 1

Cyonin assays on media which had bathed placental cells in culture for three or four days

SPECIMEN	TISSUE	DAYS CULTURED	VOL. PER RAT	OVARIAN EFFECT
		days	*ml.*	
1530	Placenta, 4 months	66	1.75	Corpora lutea
1508	Placenta, 4 months	74	1.50	Negative
1522	Placenta, 4 months	87	2.00	Follicle growth
1509	Hydatidiform mole	24	1.25	Corpora lutea
1521	Hydatidiform mole	37	1.75	Corpora lutea
1500	Combined media of above placenta and mole	61 11	4.00	Corpora lutea
1713	Chorion, 4 months	142	5.4	Negative
1714	Decidua, 4 months	142	3.00	Negative
1700	Ectopic chorion	65	2.50	Corpora lutea
1734	Ectopic chorion	109	4.00	Follicle growth
1808	Ectopic chorion	180	14.00	Corpora lutea
1596	Combined media of above chorions	59 28	3.60	Corpora lutea
1841	Placenta, 4 months	99	4.00	Negative
1866	Ectopic chorion	19	5.35	Corpora lutea
2000	Placenta, 4 months	60	3.00	Corpora lutea
2094	Placenta, 4 months	99	2.54	Follicle growth
2095	Placenta, 3 months	38	1.20	Corpora lutea
S-1	Term placenta	10	2.00	Corpora lutea
S-2	Term placenta	10	1.00	Follicle growth
S-3	Term placenta	34	2.40	Follicle growth
S-17	Term placenta	34	1.20	Negative
S-22	Term placenta	55	2.60	Follicle growth
S-25	Term placenta	75	4.50	Negative
S-27	Term placenta	85	4.20	Negative
S-26	Hydatidiform mole	7	2.00	Corpora lutea
S-28	Hydatidiform mole	10	3.25	Corpora lutea
S-30	Hydatidiform mole	31	2.40	Corpora lutea
S-31	Hydatidiform mole	62	3.00	Follicle growth?
S-24	Term placenta	16	0.75	Negative

Too much significance must not be attached to this result as it is difficult, if not indeed impossible, to isolate decidual cells in pure culture.

Consistently negative results were obtained in eighteen control assays using human placental cord serum, beef embryo extract, the whole unincubated medium and media removed from normal muscle and normal thyroid cultures. In one instance when 4.75 ml. of un-

TABLE 2

Cyonin assays on control tissue culture media

SPECIMEN	TISSUE	DAYS CULTURED	VOL. PER RAT	OVARIAN EFFECT
		years	*ml.*	
1	Human placental cord serum		4.0	Negative
2	Beef embryo extract		8.0	Negative
3	Unincubated media		3.5	Negative
1520	Unincubated media		3.0	Negative
1806	Unincubated media		2.5	Negative
2001	Unincubated media		3.6	Negative
S-0	Unincubated media		4.5	Follicle growth
S-29	Unincubated media		1.75	Negative
1502	Muscle		1.75	Negative
1507	Muscle	2	1.75	Negative
1842	Muscle	2	4.20	Negative
1849	Muscle	2	4.20	Negative
1867	Muscle	2	5.65	Negative
2231	Muscle	2	3.80	Negative
2233	Muscle	2	1.14	Negative
S-3	Thyroid	1	1.00	Negative
S-19	Thyroid	1	2.40	Negative
S-23	Thyroid	1	3.00	Negative

incubated whole medium was assayed some follicular growth was observed (table 2).

The absence of the placental hormone in the human cord serum can probably be explained by an inactivation of the hormone during the period of time which elapses between the collection of the serum and its use in the culture medium. One might expect to find some cyonin present in the cord serum since the maternal blood at term contains from 2,000 to 15,000 i.u. per liter of chorionic gonadotropin according to the Delfs method.

Estrogen determinations

Estrogen determinations on the same type of material were next attempted. It was immediately apparent that the interpretation of these results would be more difficult as the control medium was found to contain varying amounts of estrogen. This resulted from the varying concentrations of estrogen in the human cord serum used in making up the medium. A positive result could therefore be judged only by the amount of estrogen found in the placental culture medium in excess of that found in the control medium.

Six specimens were tested for estrogen by the vaginal estrous technique, all of which showed similar amounts of estrogen in both the placental and control media. It seemed that a more delicate method might be necessary in order to determine any slight increase in the estrogen content of the placental over the control media; the Astwood technique was therefore used in the remaining 10 assays.

The results of these assays are shown in table 3. It must be remembered that in the control assays the same batch of medium was employed in each case as was used for the accompanying placental specimen. It can be seen that, in five of these determinations, the estrogen values for the control and placental media are extremely close. In one specimen, D. Hi., the range of the test was exceeded for both media. In four instances the values for estrogen in the control and placental media differed. In two of these the control estrogen value exceeded the placental, and in two others the placental value exceeded the control. These results have been interpreted as due to faulty technique rather than to an actual variation in the estrogen content of the media.

As placental tissue might produce an inactive form of combined estrogen, a sample of placental fluid medium and a control sample of the same medium were hydrolyzed with 1.0 N sodium hydroxide, extracted with commercial hexane to remove any progesterone which might be present, acidified and extracted with ether. An oily solution of this ether extract was assayed and, as can be seen by the last assay in table 3, approximately equal amounts of estrogen were again found to be present in both media.

As it is presumed that the placenta also elaborates progesterone and as progesterone is known to mask an estrogen effect, if present in

sufficient quantities, a test was devised to detect the presence of small amounts of progesterone. Allen and Meyer have shown that "progestin" will prevent the estrous smear in the castrate mouse if the ratio of one rabbit unit of progesterone to one rat unit of estrogen

TABLE 3

Estrogen assays on media of placental and control tissue cultures by the Astwood Method

SPECIMEN	TISSUE	DAYS CULTURED	ESTROGEN PER MILLILITER
		days	*i.u.*
E. Se.	Term placenta	10	25
V. Wi.	Thyroid		20
E. Se.	Term placenta	35	34
V. Wi.	Thyroid		32
E. Se.	Term placenta	65	20
V. Wi.	Thyroid		>124
Hyd.	Hydatidiform mole	17	52
L. Ru.	Muscle		58
Hyd.	Hydatidiform mole	?	19
L. Ru.	Muscle		17
D. Hi.	Placenta, 4 months	51	>124
L. Ru.	Muscle		>124
Le. C.	Hydatidiform mole	14	19
L. Ru.	Muscle		>60
Le. C.	Hydatidiform mole	37	>120
L. Ru.	Muscle		22
Le. C.	Hydatidiform mole	47	53
	Unincubated medium		28
B. De.	Ectopic chorion (fluid hydrolized)	38	5
V. Wi.	Thyroid		7

is used. Astwood and Jones have shown, however, that much smaller amounts of progesterone in a solution of estrogen, although incapable of completely preventing the appearance of estrus, will produce a conversion of the estrous smear to the diestrous type if

the injections are continued over a period of 4 to 6 days. Control and placental fluid media were therefore injected into adult, castrate rats in amounts sufficient to produce vaginal estrus and injections were continued over a period of 10 days. Both control and placental test animals remained in constant vaginal estrus throughout the injection period. It was therefore assumed that there was no appreciable amount of progesterone present.

Lastly, as Heller and Heller have demonstrated that estrogen is inactivated by incubation with liver cells, it was thought necessary to determine whether placental cells growing in tissue culture might also exhibit this activity. If this were the case, the estrogen might be destroyed or inactivated as rapidly as it was produced. Accordingly, known amounts of estradiol were added to culture tubes of placental cells and also to blank tubes of identical culture medium These latter tubes were not incubated. The placental tubes with the added estradiol were incubated for the usual 4 days at 37°C. Estrogen assays were performed by the Astwood method on the unincubated culture medium and on the medium in which the placental cells had been grown. Again equal amounts of estrogen were found in the control and placental media.

SUMMARY

Human placental cells have been grown in continuous cultures in roller tubes. The specimens cultured were 5 three month placentae, 2 placentae of ectopic pregnancies, 2 full term placentae, and 3 hydatidiform moles. Aschheim-Zondek tests were performed on the supernatant cell free fluid media which were removed at different intervals after primary explantation and after having been in contact with the living placental cells for only three to four days. Fourteen tests of the twenty-nine which were performed were positive for cyonin as judged by the presence of corpora lutea in the 21 day old rat ovary. Marked increase in the size of one or more follicles was observed six times and weak follicle stimulation once. Four assays were negative. Control tests carried out on various constituents of the medium used and on media from human muscle and thyroid cultures were negative.

Ten estrogen assays, performed by the 6 hour Astwood method,

failed to demonstrate any consistent increase in the amount of estrogen present in the placental as compared with the control tissue culture medium. Hydrolysis of the fluid did not increase the estrogenic activity. Tests for progesterone failed to indicate that this hormone was present in amounts sufficient to obscure an estrogen effect. Estradiol was not destroyed by incubation with placental cells growing in tissue culture.

CONCLUSIONS

It has been demonstrated that placental cells produce cyonin, that is, chorionic gonadotropin. The type of cells apparently responsible for this activity are the Langhans' cells. These cells will produce cyonin under conditions found in continuous tissue cultures and may retain this capacity as long as six months in culture. It has not been possible to demonstrate that placental cells can produce estrogen under similar circumstances.

BIBLIOGRAPHY

ABRAMOWITZ, A. A., AND HISAW, F. L.: The effect of proteolytic enzymes on purified gonadotropic hormones. *Endocrinology*, **25:** 633, 1939.

ASCHHEIM, S.: Hormone und schwangerschaft. *Med. Klin.*, **22:** 2023, 1926.

ASCHHEIM, S., AND ZONDEK, B.: Ovarialhormon, Wachstum der Genitalien, sexuelle Frühreife. *Klin. Wchnschr.*, **5:** 2199, 1926.

ASTWOOD, E. B.: A six-hour assay for the quantitative determination of estrogen. *Endocrinology*, **23:** 25, 1938.

ASTWOOD, E. B., AND GREEP, R.: Nomenclature of gonad stimulating hormones of placental origin. *Science*, **89:** 81, 1939.

COLLIP, J. B., SELYE, H., AND THOMPSON, D. L.: Gonad stimulating hormones in hypophysectomized animals. *Nature*, **131:** 56, 1933.

DELFS, E.: An assay method for human chorionic gonadotropin. *Endocrinology*, **28:** 196, 1941.

FEVOLD, H. L.: The Gonadotropic Hormones. *Cold Spring Harbor Symposium on Quantitative Biology*. **5:** 93, 1937.

FEVOLD, H. L., AND HISAW, F. L.: Concentration of gonadotropin substance from pregnancy urine. *Proc. Soc. Exp. Biol. and Med.*, **34:** 2199, 1926.

FRIEDHEIM, E. A. H.: Die Züchtung von Menschlichem Chorionepithel in vitro. Ein Beitrag zur Lehre vom Chorionepitheliom. *Virchows Arch. f. Path. Anat.*, **272:** 217, 1929.

FRIEDHEIM, E.: La culture in vitro de villosités placentaires humaines. *Comp. rend. soc. de biol.*, **98:** 123, 1928.

GEY, G. O., AND GEY, M. K.: Maintenance of human normal cells and tumor cells in continuous tissue culture. *Am. J. Cancer*, **27:** 45, 1936.

GEY, G. O., SEEGAR, G. E., AND HELLMAN, L. M.: The production of gonadotropic substance (prolan) by placental cells in tissue culture. *Science*, **88:** 306, 1938.

GUGGISBURG, H., AND NEUWEILER, W.: Uber Züchtungsversuche von Plazenta u. Decidua. *Zentralbl. f. Gynäk.*, **50:** 1437, 1926.

GURIN, S.: Carbohydrates of the gonadotropic hormones. *Proc. Soc. Exp. Biol. and Med.*, **49:** 48, 1942.

HEIM, K.: Weitere Ergebnisse bei Auspflanzungsversuche von Plazenta u. Decidua. *Zentralbl. f. Gynäk.*, **51:** 653, 1927.

JONES, G. E. SEEGAR, AND ASTWOOD, E. B.: The physiological significance of the estrogen: progesterone ratio in vaginal cornification in the rat. *Endocrinology*, **30:** 295, 1942.

KEIBEL, F., AND MALL, F. P.: Manual of Human Embryology. J. B. Lippincott & Co., Philadelphia and London, 1910–12.

HELLER, C. G., HELLER, E. J., AND SEVERINGHAUS, E. L.: Metabolism of estrogenic hormones—in vitro studies. *Am. J. Physiol.*, **126:** P 530, 1939.

LEONARD, S. L.: Difference between human anterior pituitary extract and prolan. *Proc. Soc. Exp. Biol. and Med.*, **30:** 1251, 1933.

LI, C. H., SIMPSON, M. E., AND EVANS, H. M.: Interstitial cell stimulating hormone: method of preparation and some physico-chemical studies. *Endocrinology*, **27:** 803, 1940.

MADRUZZA, G.: Azione biologica del trapianto di decidua sulla ghiandola mamman. *Riv. ital. di ginecol.*, **6:** 113, 1927.

NEUWEILER, E.: Über Explantationsversuche menschlicher Plazenta. *Monatschr. f. Geburtsch. u. Gynäk.*, **77:** 437, 1927.

SANNICANDRO, G.: Sulle culture in vitro di placenta umana; ricerche sulle modalità di accrescimento e sulla biologia dei villi coriali. *Ann. di. ostet. e ginec.*, **56:** 3, 1934.

SENGUPTA, B.: Plazenta in der Gewebekultur. *Arch. f. exper. Zellforsch.*, **17:** 281, 1935.

TASZKAN, R.: Ein neues Züchtungsverfahren für Gewebe erwachsener Tiere u. menschliche Placenta in tierischem u. menschlichem Serum. *Virchows Arch. f. Path. Anat.*, **299:** 106, 1937.

THE EFFECT OF A PURIFIED DIET DEFICIENT IN CARBOHYDRATE ON THE RAT

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Carbohydrate is needed to furnish energy for cellular activity and muscular contraction and for the metabolism of fat. Although carbohydrate may be formed from protein and probably fat as well, the question as to whether these materials can furnish enough for the maintanence of life and growth in the complete absence of dietary carbohydrate has not been studied.

The experiment to be reported deals with such an investigation in rats which were placed on a purified diet containing no carbohydrate.

MATERIAL AND METHODS

The diet employed was as follows: purified casein, 44.55; lard (crisco), 40; purified sweet butter fat, 10; salts 5.45. Two hundred milligrams of choline hydrochloride and 15 drops of viosterol were added to each kilogram of diet. The following amounts of crystalline vitamins* were administered orally in divided doses twice a week: thiamine hydrochloride 0.2 mgm., riboflavin 0.28 mgm., calcium pantothenate, 1.0 mgm., pyridoxine 0.2 mgm. and nicotinic acid, 1.0 mgm. Alpha tocopherol, 1 mgm. was given every other week. For the control diet 10 per cent sucrose (Dyno) was substituted for 5 per cent of the protein and fat respectively. The diet and water were given *ad libitum*.

A total of 18 albino rats weighing 43–56 grams were used. Twelve were placed on the carbohydrate-free diet, while 6 were given the control diet. There were 2 females in each group. One animal of each series was autopsied after 21 days. The remaining animals, with the exception of one deficient rat which was pregnant, were sacrified after having received the diets for 77 days.

The animals were starved for 24 hours before death. They were then anesthesized with luminal; blood was removed by cardiac puncture and determinations for glucose carried out. All the tissues were removed and fixed in Zenker-formol. Before fixation the 2 adrenals and thymus were cleaned of fat and weighed. Blocks of the liver, adrenal, kidney, pancreas, thymus, thyroid, parathyroid and testis or ovary were embedded in paraffin, cut and stained with hematoxylin and eosin.

* Furnished through the courtesy of Dr. D. F. Robertson, Merck & Co.

EXPERIMENTAL RESULTS

As will be seen from the growth curves (fig. 1) the average gain in weight of the 2 groups was good. Because of the high fat content of both diets the coats of all the animals were greasy but there was no loss of hair. The animals seemed active and normal in every respect.

Both females in the carbohydrate-free group gave birth to litters of young. One of the females in the control series did likewise. The young of one of the carbohydrate deficient and of one of the control animals were found destroyed. However, one carbohydrate deficient female raised her litter and they appeared normal. One animal of the control series was found to have fetuses in utero at autopsy.

The serum glucose showed no constant relationship to the 2 types of diet. The average level in 4 controls was 74 mgm. per cent (max. 87,

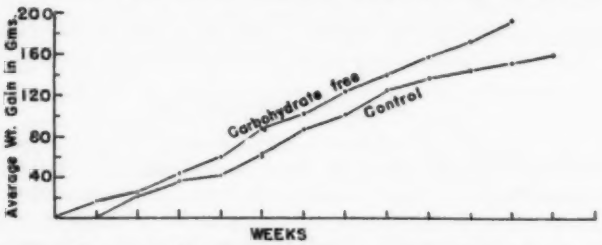

FIG. 1. GROWTH CURVES OF ANIMALS FED A CARBOHYDRATE FREE DIET COMPARED WITH THAT OF CONTROLS

min. 62) while that in the deficient rats was 87 mgm. per cent (max. 136, min. 74).

For reasons which will be stated below, special attention was given to the weights of the adrenals and the thymus. The weights of both adrenal glands and of the thymus relative to the body weight, however, were not significantly different in the two groups.

Microscopical examination of the tissues of each group of animals revealed no differences. Spermatogenesis was active in the testes. There was no excessive liver fat. No differences were found in the islets of Langerhans in the pancreas.

DISCUSSION

The observations would tend to show that carbohydrate *per se* is not necessary in the diet. Adequate growth took place in its absence.

It is thus evident that the rat, at least, can manufacture this foodstuff from protein and probably fat. Modifications of the relative amounts of the latter materials in this diet might produce alterations in growth.

The failure to find an increase in the weight of the adrenal glands of the deficient animals should be mentioned. Since it has been suggested that steroids from the adrenal cortex are important for the formation of carbohydrate from protein (1), it was thought an excessive demand on this mechanism might lead to an increase in size of the glands. Such was not the case, however, though it must be pointed out that the difference of 10 per cent carbohydrate in the diets may not have been enough to bring out any difference. It has also been observed (2) that thymic tissue undergoes atrophy when stimulated by adrenal cortical hormones; the absence of any change in our animals further strengthens the evidence of a lack of stimulation on the adrenals.

That similar results might not be encountered in other species is suggested by a report by von Chwalibogowski (3) who reared a child from birth on a "practically carbohydrate-free" diet. At 10 months of age it developed ketosis and the experiment was then stopped.

SUMMARY

In rats which were placed on a completely carbohydrate-free diet good growth was obtained. No significant changes in the adrenal glands or thymus gland: body weight ratios were found in the deficient animals as compared with the controls.

BIBLIOGRAPHY

1. Long, C. N. H.: *Endocrinology*, **30**: 870, 1942.
2. Wells, B. B. and Kendall, E. C.: *Proc. Staff Meetings Mayo Clinic*, **15**: 133, 1940.
3. von Chwalibogowski: *Acta Paediat.*, **22**: 110, 1937.

SULFANILAMIDE AS A PROPHYLACTIC AGENT IN RHEUMATIC FEVER[1]

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This investigation was undertaken to test the value of small doses of sulfanilamide in the prevention of rheumatic recurrences and to conduct concurrent bacteriological studies which might throw some light on the possible role of beta hemolytic streptococcal infections in rheumatic fever.

The problem was approached in a manner similar to that previously adopted by Thomas and France (1), Coburn and Moore (2), Kuttner (3), and Dodge, Baldwin and Weber (4); that is, the study was designed to compare the incidence of streptococcal infection and rheumatic exacerbations in a group of patients given prophylactic doses of sulfanilamide, with the incidence in a similar group of patients to whom the drug was not administered.

To this end, patients were selected for each group according to the same criteria. The prerequisites were an acute rheumatic episode within three years but no clinical or laboratory evidence of rheumatic activity for at least three months prior to commencement of the study. Furthermore, the two groups were kept comparable as far as possible with regard to the rheumatic status of the patients in each group. Thus, in each group chorea was matched against chorea, mild rheumatic disease against mild rheumatic disease, moderate against moderate, and severe infection against severe infection.

The study was carried over a two-winter period. The administration of sulfanilamide was begun late in the fall of 1940. The drug was given to the initial group of patients throughout the winter and spring and was discontinued during the summer. The following September

[1] These experimental investigations were supported in part by the John and Mary R. Markle Foundation.

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In 1941–42, Abbot Laboratories Fellow in Preventive Medicine.

(1941), prophylaxis with sulfanilamide was started again in the original group. In addition, new patients were added to the group receiving sulfanilamide and to the control group in comparable numbers and according to comparable criteria. With the exception of one patient, who was twenty years of age, the age range in both groups was from six to sixteen years.

As regards the administration of sulfanilamide, the optimum concentration of sulfanilamide in the blood was arbitrarily set at 1–2 milligrams per cent. Maintenance of this level required dosages of from 0.6 to 1.5 grams of the drug daily, depending on the age and weight of the patients.

The group of patients who received sulfanilamide were followed very closely during the first two months of drug administration. White blood cell counts, hemoglobin determinations, and urinalyses were done on the first, fifth, tenth, and fourteenth days of drug administration. From the fourteenth to the twenty-eighth days, white blood cell counts and hemoglobin determinations were done every other day and from then to the sixtieth day at weekly intervals. Thereafter physical examinations and blood and urine studies were performed at bi-monthly intervals for the remainder of the experimental period.

With these precautions, no serious reactions were encountered. A few patients, however, proved to be sensitive to the drug and had to be withdrawn from the group. Since this had been expected, the contingency had been met by placing enough additional children in the group receiving prophylactic sulfanilamide to allow for the withdrawal of a few from the group and still have the number of patients remain comparable in both the treated and control groups.

Slight weight losses and moderate decreases in hemoglobin were common in the initial stages of drug administration but acute hemolytic anemia was not encountered. During the first year the drug was withdrawn from one patient because of persistent anorexia and a borderline leukopenia (3500 W.B.C.) and in another because of a questionable recurrence of rheumatic fever on the twelfth day of the administration of sulfanilamide.

During the second year of the study, one patient developed leukopenia on the twenty-fifth day of prophylaxis but, on a reduced dosage of the drug, the white blood count rose to a low normal value and re-

mained at that level throughout the season. In four instances sulfanilamide had to be withdrawn; in two cases because of rashes, in one because of acute jaundice which may or may not have been related to drug therapy, and in one instance because of lack of cooperation.

Sedimentation rates were determined and throat cultures were obtained from all patients at regular intervals. In the group receiving sulfanilamide, however, throat cultures were taken and sedimentation rates were determined twice as frequently as in the control group. With this exception, patients in both groups were managed similarly in every detail.

Concurrently with the clinical investigation, bacteriological and immunological studies were carried out on all beta hemolytic streptococci isolated from patients in both groups. The bacteriological investigation of each strain isolated consisted in grouping the organism, and in classifying it on the basis of morphological and cultural characteristics according to the criteria of Dawson, Hobby, and Olmstead (5). In addition, all Group A strains were typed.[3]

The immunological status of the rheumatic subject with regard to the development of streptococcal antistreptolysins, antifibrinolysins, anti "M" precipitins and agglutinins has been investigated by numerous workers in this field. It seemed, therefore, of interest to investigate the antibacterial response of the blood of these rheumatic subjects to the hemolytic streptococcus. The method selected for the titration of the patient's antibacterial antibody was the bactericidal test which measures the killing power of the patient's whole blood against the streptococcus isolated from his nasopharynx. Accordingly, when a streptococcus was cultured from a patient, the bactericidal power of the patient's blood for his own organism was determined at regular intervals by this test.

METHODS

Throat cultures. A tube containing blood broth was inoculated directly from the nasopharyngeal swab. The tube was incubated at 37°C. for three to four hours, following which a loopful of the culture was streaked in a manner which gave well-isolated colonies on a blood agar plate. After overnight incubation

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[3] The authors wish to express their gratitude to Dr. Rebecca Lancefield for her kindness in typing these strains.

beta hemolytic colonies were fished from the plate, stained with Gram's stain, and if pure, were transferred to blood broth. From then on the following laboratory procedures were carried out on each strain.

Freezing and drying. To guard against dissociative changes occurring spontaneously in the organisms, immediately after isolation in pure culture, all strains were frozen and dried in vacuo according to the method of Flosdorf and Mudd (6).

Grouping. Carbohydrate extracts of each strain were prepared according to the method of Fuller (7). The extracts were then set up against Lederle grouping sera and the Group of the organism determined by means of the precipitin reaction. Initial readings were made at one hour and final readings at twenty-four hours.

Colony type. Blood broth cultures were streaked on freshly poured Dawson (5) plates so as to obtain isolated colonies. Mucoid, smooth, and rough colonies could easily be distinguished on this special medium even without the aid of a colony microscope.

Capsules. Capsule preparations were made and stained by the Seastone (8) method. Two loopfuls of a 20 per cent rabbit serum broth culture, incubated for two to three hours, were mixed with equal quantities of rabbit blood on a slide. After thorough mixing, blood smears were made in the usual manner. The slide preparations were then stained with Wright's (8) stain and examined microscopically under oil immersion. With this method large, pink staining capsules when present were easily demonstrable.

Bactericidal tests. About 8 cc. of blood was withdrawn from the patient by asceptic venapuncture and defibrinated by shaking in a flask with glass beads. Para-aminobenzoic acid in a final concentration of 0.2 mg. per cent was added to all blood specimens obtained from patients receiving sulfanilamide in order to inhibit the effect of the drug in the blood. 0.25 cc. of blood was then carefully measured into each of six sterile pyrex glass tubes 5 cm. long and 9 mm. wide. A 20 per cent rabbit-serum broth culture of the patient's organism incubated for 18 hours was diluted serially in broth from 1/10 through 1/1,000,000. Beginning with the 1/1,000,000 dilution, *one* drop of each dilution was carefully introduced with the same capillary pipette into each of the pyrex tubes containing the patient's blood. At the time the first drop of the 1/1,000,000 dilution was added to the blood, one drop of the same dilution was introduced into a sterile petri dish from the same capillary pipette. Ten cc. of melted blood agar were added to the petri dish, mixed, cooled, and incubated over night. In this way, the number of streptococci added to each tube was estimated. The pyrex tubes were sealed in an oxygen flame, put in a rotating box and incubated at 37° C. for twenty-four hours. They were then taken out of the rotating box and incubated an additional twenty-four hours without rotation. At the end of that time, the tubes were broken open, the contents well mixed with a capillary pipette, and one drop from each tube was streaked on a blood agar plate. The plates were incubated over night and the presence or absence of growth was noted on the following day.

Parallel bactericidal tests were run simultaneously on strains cultured repeatedly from the same patient. Thus in the case of a given strain—for example, #37, which was isolated three times—37 a, b, and c cultures were set up against the patient's blood at each bleeding. The complete correspondence of results in each series served as an additional check on the identity of the strains.

RESULTS

During the first year of the study, eighteen patients received prophylaxis with sulfanilamide. The drug was withdrawn from one patient because of a toxic reaction and in one other instance because of a questionable recurrence of rheumatic fever which developed within the first two weeks of drug administration. The remaining sixteen patients who received sulfanilamide throughout the year showed no evidence of rheumatic activity. Of the seventeen patients in the corresponding control group, five had recurrence of rheumatic fever. Two of the exacerbations were severe and three were mild.

Streptococci were isolated from five patients in the sulfanilamide group. Three were Group A and two were Group C strains. According to colony type, the three A strains, #19, 29, and 37, were intermediate between smooth and mucoid. All three were non-encapsulated. Two of the strains, #29 and #37, were type specific, being types C95 and Henson (?) respectively. The third, #19, did not fall into any of the Lancefield types. Both of the C strains were smooth. None of the five patients from whom these strains were isolated showed clinical evidence of streptococcal infection at the time the organisms were obtained and none of them developed rheumatic exacerbations subsequently. The bacteriological findings are summarized in table 1.

In the control group, streptococci were isolated from four patients. All four strains belonged in Group A. Two of the four, #6 and #30, according to colony type fell into the smooth category, were non-encapsulated and could not be typed; one, #32, intermediate between smooth and mucoid, was non-encapsulated and could not be typed. None of the three children from whom these three strains were isolated showed any clinical evidence of streptococcal infection, but one of the three patients, #30, had a mild attack of chorea during the period that the streptococcus was cultured from his throat. In the fourth instance, a fully encapsulated, mucoid, type 30 strain was isolated from a patient, #18, who had tonsillitis and cervical adenitis. Five

days later the patient developed scarlet fever and subsequently came down with a severe recurrence of rheumatic fever which persisted throughout the period of observation.

There were three other patients in the control group who had recurrences of rheumatic fever but from whom streptococci were not isolated. In two instances the bacteriological studies prior to recurrence were incomplete and therefore the occurrence of streptococci cannot be excluded. The third patient had an attack of bronchitis three weeks prior to the rheumatic exacerbation, but no streptococci were either isolated during the bronchitis or at the time of onset of the rheumatic flare-up.

Brief summaries of the chief findings in each of the five control cases are presented below.

Mild rheumatic recurrences. Patient #2, a six year old white girl who gave a definite familial history of rheumatic fever, was first seen in the Harriet Lane Cardiac Clinic in June 1938, at which time a diagnosis of rheumatic fever was made. No evidence of rheumatic heart disease was found. The patient was sent to St. Gabriel's Convalescent Home where she remained until November 1938. Thereafter the patient showed no signs of rheumatic activity up to the time of commencement of this study in December 1940. In February 1941, the patient developed bronchitis. Three weeks later, in March 1941, she was brought to the Cardiac Clinic because of joint pains, weight loss and the history of a fleeting rash. At that time the sedimentation rate was moderately elevated. The heart, although still negative except for a functional murmur, was found to be slightly larger by teleoroentgenogram than at the previous examination. The patient was again sent to St. Gabriel's where she remained until March 1942. Throat cultures taken during the attack of bronchitis and at the onset of the rheumatic recurrence were negative for beta hemolytic streptococci.

Patient #30, a twelve year old white boy, was first admitted to the Cardiac Clinic in January 1938, with a diagnosis of acute Sydenham's chorea. In February 1939, the patient had a recurrence of chorea accompanied by a transient attack of rheumatic arthritis. He was admitted to the Harriet Lane Home for treatment. After six weeks hospitalization, he was discharged greatly improved. In May 1940, the patient again developed chorea and for the first time showed signs of early rheumatic myocarditis. He was admitted to the Baltimore City Hospital where he remained until July 1940. After discharge he continued well and he was included in this study in November 1940. Toward the end of January 1941, the patient was found to have a markedly elevated sedimentation rate, and on February 7, 1941, developed a mild recurrence of chorea. He was kept in bed for two months after which he was gradully permitted to return to normal activities.

Throat cultures taken at the onset of the recurrence of chorea were positive for beta hemolytic streptococci (see Table 1).

Patient #38, an eleven year old white girl, and sister of patient #28 (see below), was first found to have rheumatic fever and rheumatic heart disease with mitral insufficiency in 1936. In May 1937, the patient had a moderately severe recurrence of rheumatic fever and rheumatic myocarditis. She then remained well until after commencement of this study in November 1940. In January 1941, the patient was thought to be developing aortic insufficiency and was put at complete rest in bed. Although the aortic diastolic murmur disappeared, the character of the apical murmur and the sedimentation rate suggested continued activity of the rheumatic infection for the next three months. Throat cultures taken two months prior to onset and at onset of the exacerbation were negative for beta hemolytic streptococci.

Severe rheumatic recurrences. Patient #18, a seven year old colored girl, was admitted to the Cardiac Clinic in 1937 with a diagnosis of acute rheumatic fever and rheumatic heart disease, with mitral insufficiency. In 1939, the patient had an exacerbation of rheumatic polyarthritis which gradually subsided over a period of five months. She then remained well until May 1941, when she developed pharyngitis and cervical adenitis. Five days later she was admitted to Sydenham Hospital with a diagnosis of scarlet fever. Two weeks after discharge from Sydenham in June 1941, there was a severe recurrence of rheumatic polyarthritis followed by a moderately severe attack of chorea which first appeared in August 1941. At the end of the experimental period this child still showed signs of persistent low grade rheumatic activity. At the time of onset of pharyngitis and cervical adenitis, a mucoid, type 30, beta hemolytic streptococcus was isolated from the patient's throat (see table 1).

Patient #28, a thirteen year old white boy with a definite familial history of rheumatic fever, was first seen in the Cardiac Clinic in 1936. On the basis of a history of repeated attacks of severe joint pains in 1933, 1934 and in 1936 plus physical signs of rheumatic heart disease, with mitral insufficiency, a diagnosis of rheumatic fever was made. After a prolonged period of bed rest (which carried over into 1937) the patient gradually recovered and his infection remained clinically inactive until January 1941. At this time the patient was brought to the Cardiac Clinic because of a severe attack of rheumatic polyarthritis and signs of rheumatic myocarditis. After prolonged bed rest he gradually recovered over an eight month period. Throat cultures taken after onset of the rheumatic exacerbation were negative for beta hemolytic streptococci. It is unfortunate that, owing to lack of cooperation on the part of the patient, throat cultures prior to the recurrence were not obtained.

In the 1941–42 period, the clinical and bacteriological results are given to June 1, 1942. A total of twenty-nine patients were given sulfanilamide at first. The drug was discontinued in three instances,

however, because of toxic reactions and in one instance because the patient did not cooperate. Of the remaining twenty-five patients who continued to take the drug, one patient developed very questionable signs of rheumatic activity; that is, a faint diastolic murmur, not heard by all observers, reappeared but there was no other clinical or laboratory evidence of exacerbation. In the corresponding control group of twenty-four patients, rheumatic exacerbations did not occur throughout the year. Streptococci, however, were isolated from three patients in the control group. All three strains belonged in Group A: One, #56, was smooth and non-encapsulated; one, #42, was intermediate between smooth and mucoid and non-encapsulated; the third, #26, was a fully encapsulated mucoid strain. To date, rheumatic recurrences have not been observed in the patients from whom these strains were isolated.

The bactericidal findings are presented in table 1. The possible significance of these data will be considered in the following discussion.

DISCUSSION

With regard to the value of sulfanilamide as a prophylactic agent in rheumatic fever, it is noteworthy that out of a total of forty-one patient-seasons over a two-year period, only one patient receiving sulfanilamide developed a questionable recurrence of rheumatic fever. Among the controls in the corresponding period of time, out of a total of forty-one patient-seasons, there were five recurrences of rheumatic fever, or over ten per cent, and two of the attacks extended into the second season. The data on these observations are summarized in table 2.

Concerning the bacteriological studies, the data presented are insufficient to allow any conclusions to be drawn regarding the occurrence, group, type, or cultural characteristics of hemolytic streptococci in relation to rheumatic exacerbations. In the sulfanilamide group, three patients harbored Group A streptococci for considerable periods of time. Antibodies, as evidenced by the bactericidal test, were present for prolonged periods in two of these three patients. Homologous antibodies were also present in two patients who harbored Group C organisms over a period of time.

TABLE 1

Characteristics of the organisms and bactericidal power of the patients' blood in the nine cases in which beta hemolytic streptococci were found in the 1940–1941 season

PATIENT AND STRAIN NUMBER	BETA HEMOLYTIC STREPTOCOCCUS						BACTERICIDAL POWER: NUMBER OF ORGANISMS KILLED PER CC. OF PATIENT'S BLOOD								CLINICAL STATUS
	Group	Type	Capsule	Growth in broth	Appearance of colonies	Times isolated	Nov.	Dec.	Jan.	Feb.	March	April	May	June	
Sulfanilamide															
9	C	Not typed	0	Flocculent	Smooth	3	10^{5}*	0*	0*	Leukopenia—off drug					Well
19	A	Not classifiable	0	Diffuse	Between smooth & mucoid	1	10^{3}*	10^{4}†	0†	10^{3}†	0†	†	†		Well
29	A	C 95	0	Granulo-diffuse	Between smooth & mucoid	4	*	†	0*, 10^{9}	10^{3}*	10^{4}*	0†	†		Well
37	A	Henson?	0	Granulo-diffuse	Between smooth & mucoid	3	†	†	†	†	10^{4}*, 10^{3}*	0*	0†		Well
39	C	Not typed	0	Flocculent	Smooth	3	†	10^{4}*	10^{3}*	10^{4}*	10^{3}†	0†			Well
Control															
6	A	Not classifiable	0	Flocculent	Smooth	1	*	10^{3}†	†	10^{3}†	0†	0†	†		Well
18	A	30	++++	Diffuse	Mucoid	1	†	†	†	†	†	†	*	0†	Severe rheumatic recurrence

TABLE 1—(*Concluded*)

30	A	Not classifiable	0	Flocculent	Smooth	1	†	†	†	*	0†, 10^4†	0†	†		Mild attack chorea
32	A	Not classifiable	0	Granulo-diffuse	Between smooth & mucoid	1	†	†	†	†	*			10^3†	Moved away

* Represents occasions on which beta hemolytic streptococci were isolated from patients' throats.

† Represents throat cultures negative for beta hemolytic streptococci.

Note: Bactericidal titres of 10^2 or less are not considered significant in measuring the bactericidal power of blood.

From the seven patients of the control group who harbored streptococci at some time during the two-season period, the organisms could only be isolated once in each instance. Of the seven, those patients who showed evidence of bactericidal antibodies developed low titres of antibody for very brief periods. The explanation for these observations is not apparent.

No conclusions are possible from these bacteriological data, but these observations suggest a method of study whereby an investigation might be undertaken on a larger series of patients. With regard to the clinical findings presented, the authors are fully aware of the fact that the number of patients comprising the study is too small to be of any statistical significance. As originally planned, the study was to

TABLE 2

Total number of treated and control cases and number of rheumatic recurrences in each group

YEAR	SULFANILAMIDE GROUP				CONTROL GROUP		
	Number of patients		Rheumatic recurrence		Number of patients	Rheumatic recurrence	
	Started	Finished	Mild	Severe		Mild	Severe
1940–41	18	16	0	0	17	3	2
1941–42	29	25	1?*	0	24	0	0
Total patient-seasons ..		41	1	0	41	3	2

* This case occurred among the nine new patients added to the sulfanilamide group in 1941.

have extended over a five year period and new patients were to have been added to the series each successive year. Owing to the present circumstances, however, the investigation had to be discontinued at the end of the second year. These observations are, therefore, of necessity, incomplete. It was felt, however, that since they tend to corroborate the findings of other investigators (1, 2, 3, 4), it would be of interest to report them.

SUMMARY

1. Out of 16 patients receiving sulfanilamide as a prophylactic for two seasons and 9 patients so treated for one season (41 "patient-seasons"), a questionable recurrence of rheumatic fever was observed in one case.

2. In a comparable group of patients, 17 of whom were observed for two seasons and 7 for one season, there were five recurrences of rheumatic fever.

3. Studies of the beta hemolytic streptococci isolated from the patients' throats were made in conjunction with the clinical study.

BIBLIOGRAPHY

1. Thomas, C. B., and France, R.: *Bull. Johns Hopkins Hosp.*, 1939, **64:** 67.
2. Coburn, A. F., and Moore, L. V.: *J. Clin. Invest.*, 1939, **18:** 147.
3. Kuttner, A. G.: In press.
4. Dodge, K., Baldwin, J., and Weber, M. Personal Communication.
5. Dawson, M. H., Hobby, G. L., and Olmstead M.: *J. Infect. Dis.* 1938, **62:** 138.
6. Flosdorf, E. W., and Mudd, S.: *J. Immunol.*, 1938, **34:** 469.
7. Fuller, A. T.: Brit. *J. Exp. Path.*, 1938, **19:** 130.
8. Seastone, C. V.: *J. Bact.*, 1934, **28:** 481.
9. Strauss, E., Lowell, F. C., and Finland, M.: *J. Clin. Invest.*, 1941, **20:** 189.

A STUDY OF THORACIC DUCT LYMPH IN EXPERIMENTAL CRUSH INJURY AND INJURY PRODUCED BY GROSS TRAUMA

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The present world conflict has stimulated renewed interest in crush injuries. This type of injury results in disturbance to the circulation of the part as well as gross trauma to the tissues.

The present study was an attempt to determine whether or not the thoracic duct lymph which is obtained from an animal with a crush injury has effects when injected into a recipient which are different from those which follow the injection of lymph from an uninjured control animal. Since the results showed a difference, the effects of injecting lymph which was obtained from animals with gross trauma to an extremity were determined subsequently.

METHODS AND RESULTS

All of the experiments were performed on dogs. Pain was prevented in both the donors and the recipients of the lymph by the administration of morphine and nembutal, the dose of morphine being .016 gm. per kilogram of body weight and that of nembutal being .020 gm. per kilogram. In all instances the lymph was collected by cannulation of the thoracic duct. Clotting was prevented by a small amount of heparin inserted in the cannula. The donors of the lymph were larger than the recipients, the reason for this being that it seemed desirable to magnify any possible ill effects. The lymph from both the controls and the experimental animals was filtered through several thicknesses of gauze, placed in a cooling unit overnight, and administered to the recipient the following morning. The time used for the intravenous injection of the lymph, which in most instances was 100 cc. or less, was approximately 15 minutes. If the animal was exhibiting an unfavorable reaction, such as a decline in blood pressure, the lymph was injected more slowly.

The observations which were made on the recipients of the lymph included the femoral arterial pressure by the needle puncture method, the hematocrit reading, and a microscopic study of the urine. Urine was obtained by the insertion of an indwelling urethral catheter. In many instances the control specimen of urine showed a few red blood cells, probably due to trauma from the catheter.

Control Studies. Twelve control experiments were performed. The collection of lymph was begun approximately one hour after the anesthetic had been administered and was continued for periods ranging from 9 to 12 hours. The quantity of lymph which was collected varied from 86 to 234 cc. The flow was somewhat more rapid during the early part of the collection period than it was subsequently.

The injection of this lymph into the recipient did not result in any instance in striking alteration of the arterial pressure. There was a moderate increase in the hematocrit reading in 8 of the 12 animals. As stated previously, a few red blood cells were usually present in the control specimen of urine which was obtained after catheterization. A decided increase in the number of red blood cells in the urine was noted in some of the animals following the injection of lymph. It is possible but unlikely that this increase was due to trauma from the catheter. Casts were observed in the urine of one dog. All animals survived with the exception of one which died 40 hours after the beginning of the experiment. Congestion of the lungs and a small quantity of blood tinged fluid in each pleural cavity were seen at autopsy. The results of these experiments are given in Table I.

Crush Injury. Twelve such experiments were performed. Crush injury was produced in the donor of the lymph by the application of a press to one of the posterior extremities. The method has been described in detail by Duncan and Blalock (1). The apparatus was applied at a pressure of approximately 500 pounds and was left in place for 5 hours. About one hour before the press was removed the thoracic duct was cannulated and the collection of lymph begun. The rate of lymph flow was slightly less during the hour after removal of the press than it had been during the preceding hour and there was a progressive decline thereafter. Death resulted in all the animals which received the crush injury, the survival time after removal of the press varying from 3 to 19 hours. The lymph was collected during the entire period extending from removal of the press until death in all instances except one. In this experiment the collection was terminated after 8 hours. The quantity of lymph which was obtained ranged from 27 to 115 cc. In each instance most of this lymph was injected into a recipient.

The injection of the lymph into the recipient was followed by an early significant decline in blood pressure in 5 of the 12 animals. This decline varied from 22 to 60 mm. of mercury. Three of the 5 animals died at intervals ranging from 4 to 18 hours. There was one other death; this animal did not have an early alteration in blood pressure following injection of the lymph. Marked congestion of the lungs was observed in 2 of the 4 animals which died.

TABLE I

The Effects of the Injection of Lymph which was Obtained from Control Animals

EXP. NO.	DONOR WT.	TIME OF COLLECTION	QUANTITY LYMPH	RECIPIENT WEIGHT	LYMPH INJECTED	EFFECT OF INJECTION ON BLOOD PRESSURE	HEMATOCRIT READING		URINE—MICROSCOPIC STUDY—HOURS AFTER INJECTION		
							Control	6 hours	2 hrs.	6 hrs.	24 hrs.
	kg.		*cc.*	*kg.*	*cc.*						
1	10	11 hr.	105	6.0	100	No change	42.4	46.6	No casts	No casts	
2*	11.6	10 hr. 30 min.	122	6.1	110	5 mm. rise	32.3	39.7	No casts	No casts	
3	15.5	9 hr. 20 min.	86	6.7	82	No change	54.2	60.4	No casts	No casts	No casts
4	10.2	11 hr. 45 min.	145.5	7.3	138	No change	46.4	55.0			
5	10.5	9 hr.	91.0	6.2	90	15 mm. rise	42.0	46.7	No casts	No casts	No casts
6	21.4	10 hr.	234	5.2	109	5 mm. rise	46.3	55.6	No casts	No casts	No casts
7	21.4	10 hr.	234	6.3	110	10 mm. rise	36.0	30.5	No casts	No casts	No casts
8	13.0	10 hr.	225	5.5	111	No change	45.4	45.2	No casts	No casts	No casts
9	13.0	10 hr.	225	6.1	110	5 mm. rise	44.7	45.0	No casts	No casts	No casts
10	13.5	10 hr.	142	6.7	137	No change	44.3	48.5	No casts	No casts	No casts
11	13.5	11 hr.	192	7.2	150	10 mm. rise	51.5	55.4	1 granular cast	Few casts	No casts
12	10.0	11 hr.	178	6.9	170	15 mm. rise	49.0	47.4	No casts	No casts	No casts

* Dog died 40 hours after beginning of experiment. Congestion of lungs and small quantity of blood tinged fluid in each pleural cavity.

A moderate increase in the hematocrit reading was noted in all experiments. The number of red blood cells in the urine was approximately the same as in the recipients of the control lymph. On the other hand, casts were found in the urine of 8 of the 12 animals which received lymph from the animals with crush injury. The results of these experiments are given in Table II.

Gross Trauma to an Extremity. Ten experiments of this type were performed. One of the posterior extremities of the anesthetized animal was struck many blows with a blunt instrument for approximately five minutes. The femur was not broken. The collection of lymph was begun at the time that the trauma was inflicted.

TABLE II

The Effects of the Injection of Lymph which was Obtained from Animals with Crush Injury

EXP. NO.	DONOR WT.	SURVIVAL TIME OF DONOR AFTER PRESS REMOVED	QUANTITY LYMPH	RECIPIENT WT.	LYMPH INJECTED	EFFECT OF INJECTION ON BLOOD PRESSURE	HEMATOCRIT READING		URINE—MICROSCOPIC STUDY—HOURS AFTER INJECTION			COMMENT
							Control	6 hours	2 hours	6 hours	24 hours	
	kg.		*cc.*	*kg.*	*cc.*	*mm. Hg*						
1	21.0	5 hr. 20 min.	75	5.1	75	5–10 rise			Few casts			Recipient survived
2	9.3	2 hr. 55 min.	27	6.7	20	10–15 rise	46	52.7	Few casts	Few casts		Recipient survived
3	12.7	11 hr.	60	6.2	55	22 fall	37.6	39.7	No casts	No casts		Recipient survived
4	13.3	3 hr. 50 min.	30.5	8.5	30.5	5–10 rise	41.0	46.8	No casts	No casts	No casts	Recipient survived
5	13.3	16 hr.	67.5	5.5	63.0	10 fall	43.0	43.7	No casts	No casts	No casts	Recipient survived
6	18.8	19 hr. 10 min.	97.5	4.3	97.0	5 rise	38.2	41.0	Rare casts	4–7 casts		Few casts control urine. Recipient died within 18 hrs. Marked congestion of both lungs
7	21.4	4 hr. 55 min.	28	4.4	28.0	30–35 fall	39.0	55.3	Rare casts	Many casts		Recipient died within 18 hrs. Marked congestion of both lungs
8	19.6	6 hr. 15 min.	64.5	5.0	60.0	60 fall	47.2	55.0	No casts	No casts		Recipient survived
9	11.4	5 hr. 15 min.	45.5	9.0	40.0	No change	37.3	45.5	No casts	Few casts		Recipient survived
10	11.2	7 hr. 30 min.	29	5.5	28.0	30 fall	47.4	49.5	Many casts	Few casts		Recipient died within 18 hrs. Marked congestion of lungs
11	20.0	13 hr.	115	6.2	98.0	60 fall	41.2	44.0	Many casts	Many casts		Few casts in control urine. Recipient died 4½ hrs. after injection. Hematoma thigh at site of needle puncture partially responsible
12	12.5	4 hr.	32	5.7	32.0	No change	38.8	40.5	Many casts	Few casts	No casts	Recipient survived

TABLE III

The Effects of the Injection of Lymph which was Obtained from Animals with Leg Trauma

EXP. NO.	DONOR WT.	SURVIVAL TIME OF DONOR AFTER TRAUMA	QUANTITY LYMPH	RECIPIENT WT.	LYMPH INJECTED	EFFECT OF INJECTION ON BLOOD PRESSURE	HEMATOCRIT READING			URINE—MICROSCOPIC STUDY—HOURS AFTER INJECTION			COMMENT
							Control	6 hours	24 hours	2 hours	6 hours	24 hours	
	kg.		*cc.*	*kg.*	*cc.*	*mm. Hg.*							
1	15.5	5 hr. 30 min.	53	5.8	53	5 rise	45	45.8	47.4	No casts	No casts	No casts	Recipient recovered
2	12.0	11 hr. 30 min.	80	5.3	75	15 rise	41.2	43.5	41.2	No casts	No casts	No casts	Recipient recovered
3	14.2	17 hr.	71	5.5	70	20 rise	43.6	44.8	39.6	No casts	Few casts	No casts	Recipient recovered
4	15.0	14 hr. 30 min.	111	5.8	100	20 rise	39.0	49.2		No casts	Few casts	Few casts	Dog had diarrhoea. Died 24 hrs. after trauma. Autopsy did not reveal cause
5	13.4	17 hr.	125	6.9	100	10 rise	35.4	41.8	33.2	No casts	No casts	No casts	Recipient recovered
6	12.0	9 hr. 30 min.	82	5.9	79	15 rise	33.2	37.8	34.0	No casts	No casts	No casts	Recipient recovered
7	12.3	15 hr.	90	7.2	89	10 rise	44.4	48.0	41.8	No casts	No casts	No casts	Recipient recovered
8	12.0	14 hr.	117	5.8	110	15 rise	34.6	33.0	37.6	No casts	No casts	No casts	Recipient recovered
9	11.5	17 hr.	88	6.3	85	20 rise	49.6	53.8	47.4	Few casts	Few casts	No casts	Recipient recovered
10	11.3	19 hr.	76	7.3	75	10 decline	37.4	47.2		Few casts	No casts	Few casts	Few casts in control urine specimen. Died 20 hrs. after trauma. Congestion and infarcts of lungs

The quantity of lymph that was injected into the different recipients ranged from 53 to 110 cc. A slight elevation in blood pressure occurred during and shortly after the injection of lymph in 9 of the 10 animals. In the remaining animal a fall of 10 mm. of mercury occurred during the injection; six hours later the pressure was 40 mm. of mercury lower than the control observation. This animal died 14 hours later. Alterations in the hematocrit readings were pronounced in only 2 experiments. An increase in concentration of red blood corpuscles was noted in both of the animals, which were the only 2 which did not recover. The number of red blood cells in the urine was approximately the same as was observed in the control experiments and in those on crush injuries. A few casts were found in the urine in 4 animals; in one of these, casts were present before the traumatization.

As has been stated, 2 of the 10 animals died. The first of these was a weak looking animal in which soft bowel movements containing blood were noticed two hours after injection of the lymph. This animal died 24 hours after the trauma. Autopsy did not disclose the cause of death. The second animal which died was the one in which there had been a decline in blood pressure following the injection. Marked congestion of the lungs was observed at autopsy.

COMMENT

A comparison of the control experiments and those in which crush injury was produced suggests very strongly that the thoracic lymph of an animal which has had a "press" applied to an extremity for a 5 hour period contains some injurious substances which are not usually present in lymph from normal dogs. At least it can be stated that the intravenous administration of lymph obtained from animals with crush injuries is much more apt to cause a decline in blood pressure, the appearance of casts in the urine, and the death of the recipient than is lymph which is obtained from control animals. Drinker and his associates (2) have made many such injections of normal thoracic duct lymph and have found it non-toxic. The present experiments indicate that lymph from normal dogs is comparatively non-toxic, but it is to be noted that one of the recipients died. It is possible that this death was not related to the injection of lymph.

The present experiments do not allow one to be so certain regarding the presence or absence of toxic products in the thoracic duct lymph of dogs in which an extremity has been traumatized. This type of trauma differs from that in crush injuries in a number of respects, but mainly in that local ischemia and anemia are not added to the tissue damage. Previously reported experiments (3) show that the general effects of the two types of injury may be distinctly different. The results of the present experiments, in which lymph from animals with gross trauma to an extremity was injected into other animals, are intermediate between those of the control series and those in which crush injury was produced, but more nearly approach the results in the controls.

The question arises how much of the lymph from a posterior extremity enters the left thoracic duct. This question cannot be answered on the basis of the present experiments. Drinker (2) states that most of the lymph from all the extremities enters the thoracic duct. It was noted in the present experiments on crush injury and on trauma to an extremity that the lymph became pink in color.

The most plausible explanation for the untoward reactions observed in some of the animals which received lymph from donors in which crush injury had been produced is that toxic products were formed in the injured and ischemic extremity and that at least some of these entered the lymphatic vessels. It is possible but not likely that the prolonged period of low blood pressure in the donors resulted in alterations which were reflected in the lymph. If the period of low blood pressure had been the main causative agent, however, observations similar to those in crush injuries would have been noted in the experiments in which gross trauma was produced. Unreported observations (2) by Drinker and his associates on burns will probably aid in the interpretation of some of these findings.

SUMMARY

Crush injury was produced in anesthetized dogs by applying a mechanical press to a posterior extremity for 5 hours. The thoracic duct lymph which was collected after removal of the press caused ill effects in some of the animals into which it was injected, thus indi-

cating that it contained toxic products. The evidence is less clear regarding the properties of lymph which was obtained from animals with trauma to an extremity but without the added element of ischemia caused by the press.

REFERENCES

1. DUNCAN, G. W., AND BLALOCK, A. The uniform production of experimental shock by crush injury. Possible relationship to clinical crush syndrome. *Ann. Surg.*, 1942, **115:** 684.
2. DRINKER, C. K. Personal communication, 1942.
3. BLALOCK, A., AND DUNCAN, G. W. Traumatic shock—a consideration of several types of injuries. *Surg. Gyn. Obst.*, 1942, **75:** 401.

BOOK REVIEWS

(These reviews represent the individual opinions of the reviewers and not necessarily those of the members of the Editorial Board of this Journal.)

The Biological Action of the Vitamins. A Symposium. Edited by E. A. Evans, Jr., pp. 227. $3.00. *University of Chicago Press, 1942.*

This volume, devoted largely to the B vitamins, consists of essays given at the University of Chicago sesquicentennial celebration in September, 1941. The essayists include Elvehjem, Ochoa, Jolliffe, Gyorgy, Sebrell, D. T. Smith, R. J. Williams, Lepkovsky, Gordon, du Vigneaud and Griffith. It is probably the most complete and up-to-date survey that has appeared in English and will be warmly welcomed by clinicians and biochemists alike.

L. E. H., Jr.

Subacute Bacterial Endocarditis. By Emanuel Libman and Charles K. Friedberg. 108 pp. Illus. $2.75. Oxford University Press, New York, 1941.

Probably Dr. Libman and Dr. Friedberg have seen more patients suffering from subacute bacterial endocarditis than anyone else in the country. "By 1925 we had seen 800 cases, and we stopped counting them a number of years ago when the figure was 1200." The present small volume reflects this wealth of experience, so that for the reader the disease becomes vivid and three-dimensional. The book is clearly and concisely written, with excellent illustrations, including one in color of the Janeway lesions on palms and soles. The final sections on prognosis and treatment sum up the literature on the most recent methods of combatting subacute bacterial endocarditis. It is made clear that no satisfactory therapeutic measures have yet appeared, despite a slight increase in the number of recoveries when the sulfonamides are used.

C. B. T.

War Gases. By Morris B. Jacobs. 180 pp. Diagrams. $3.00. Interscience Publishers, New York, 1942.

This small volume presents in a precise yet readable form a compilation of a large amount of practical and technical information concerning the important war gases. These include the lung irritants, the lachrimators, sternutators (sneezing gases), labyrinthic gases, vesicants, systemic poisons and paralysants, screening smokes and incendiaries. It may be fortunate that of the 3000 gases which have been investigated for their value in warfare, only 38 were actually used in the War of 1914–1918, and of these only 6 are of really great importance.

The first portion of the book is of particular interest to the physician, for it deals with the physical properties and pharmacologic aspects of the gases, and their effects on materials, water, food, crops and live stock. The later sections deal in

detail with methods of detection and decontamination. An aspect of gas warfare which would be of great interest to physicians but, unfortunately, is not discussed is the medical care of patients after they have been exposed to the gases. However, references to other sources of information are given.

An inescapable conclusion after reading this book is that untrained individuals acting independently during or after gas attacks are more likely to do harm than good. The only adequate defense against gas warfare appears to be a well trained and equipped personnel. This book should serve as an admirable text for such training.

L. K. A.

Short History of Cardiology. By JAMES B. HERRICK. 258 pp. Illus. $3.50. Charles C. Thomas, Springfield, Illinois, 1942.

This book has particular interest since the author has himself contributed to medical history by his masterly description of the clinical aspects of coronary occlusion. The general plan in this volume has been to deal with historical periods and countries, but the real interest lies in the discussions of individuals, the descriptions of their personalities and their relationships with their fellow men, the evaluation of their writings and their influence on the development of the knowledge of disease. Thus the effect is achieved of showing them not only as scholars but also as men.

Although Doctor Herrick does not, unfortunately, discuss his own work, he does nevertheless throw considerable light on the reasons why coronary occlusion had not been recognized clinically before in spite of the excellent pathologic and experimental background which had long been prepared for it. The style throughout is delightful and the reproductions are excellent.

L. K. A.

Advances in Pediatrics, volume 1. Edited by ADOLPH G. DE SANCTIS. 306 pp. Illus. $4.50. Interscience Publishers, New York, 1942.

"Advances in Pediatrics" (Interscience Publishers, Inc., New York, N. Y., 1942) is the first of a projected annual series of books designed to keep the average physician acquainted with new developments in the field. No attempt is made to review the entire current literature. Instead, the book contains a group of monographs by individuals who are active in the particular field. As would be expected under such circumstances, no consistent viewpoint is maintained throughout the book. One chapter may be presented in a simple, didactic style with little emphasis on underlying causes, another as a critical review, and others as a discussion of the problem from the background of the author's original contributions to the subject. Although some sections are distinctly better than others, on the whole the book offers a ready means of bringing one's knowledge up to date on the subjects of toxoplasma, virus diseases, chemotherapy, electro-encephalography, vitamin K, surgical treatment of persistent ductus arteriosus, the premature infant, tuberculosis, and endocrinology.

E. M. B.

BOOKS RECEIVED FOR REVIEW

Ophthalmology and Otolaryngology. Prepared and Edited by the Subcommittees on Ophthalmology and Otolaryngology of the Committee on Surgery of the Division of Medical Sciences of the National Research Council. 331 pp. Illus. $4.00. W. B. Saunders Company, Philadelphia & London, 1942. (Military Surgical Manuals, Vol. 2.)

Abdominal and Genito-Urinary Injuries. Prepared under the Auspices of the Committee on Surgery of the Division of Medical Sciences of the National Research Council. 243 pp. Illus. $3.00. W. B. Saunders Company, Philadelphia & London, 1942. (Military Surgical Manuals, Vol. 3.)

First Aid, Surgical and Medical. By WARREN H. COLE and CHARLES B. PUESTOW. 374 pp. Illus. $3.00. D. Appleton-Century Company, New York, 1942.

Traumatic Surgery of the Jaws, Including First-Aid Treatment. By KURT H. THOMA. 315 pp. Illus. $6.00. The C. V. Mosby Company, St. Louis, 1942.

Clinical Anesthesia. By JOHN S. LUNDY. 771 pp. Illus. $9.00. W. B. Saunders Company, Philadelphia and London, 1942.

Formulary and Handbook. The Johns Hopkins Hospital. Edited by JOHN C. KRANTZ, JR. 253 pp. $2.00. John D. Lucas Company, Baltimore, 1942.

Introduction to Materia Medica and Pharmacology. By HUGH ALISTER MCGUIGAN and ELSIE E. KRUG. 3rd ed. 779 pp. Illus. $3.50. The C. V. Mosby Company, St. Louis, 1942.

Handbook of the Lying-In Hospital, Woman's Clinic of the New York Hospital. 158 pp. $2.00. Woman's Clinic of the New York Hospital, New York, 1942.

Changes in the Knee Joint at Various Ages. By GRANVILLE A. BENNETT, HANS WAINE, and WALTER BAUER. 97 pp. 31 pl. $2.50. The Commonwealth Fund, New York, 1942.

Surgical Pathology. By WILLIAM BOYD. 5th ed. 843 pp. Illus. $10.00. W. B. Saunders Company, Philadelphia & London, 1942.

Osler's Principles and Practice of Medicine. 14th, semicentennial (1892–1942) edition. By HENRY A. CHRISTIAN. 1475 pp. $9.50. D. Appleton-Century Company, New York and London, 1942.

Manual of Dermatology. Issued under the Auspices of the Committee on Medicine of the Division of Medical Sciences of the National Research Council. By DONALD M. PILLSBURY, MARION B. SULZBERGER, and CLARENCE S. LIVINGOOD. 421 pp. Illus. $2.00. W. B. Saunders Company, Philadelphia & London, 1942. (Military Medical Manuals.)

Fundamentals of Psychiatry. By EDWARD A. STRECKER. 201 pp. Illus. $3.00. J. B. Lippincott Company, Philadelphia, 1942.

Practical Survey of Chemistry and Metabolism of the Skin. By MORRIS MARKOWITZ. 196 pp. $3.50. The Blakiston Company, Philadelphia, 1942.